CALCIUM INDEPENDENT PHOSPHOLIPASE $A_2\gamma$ POLYNUCLEOTIDES AND POLYPEPTIDES AND METHODS THEREFOR

CROSS-REFERENCE TO RELATED APPLICATIONS

This Application is a continuation in part of pending U.S. Application No. 09/168,623 filed July 18, 2000, which is incorporated in its entirety by reference.

REFERENCE TO GOVERNMENT GRANT

[0001] This invention was made with U.S. government support under National Institute of Health Grant Numbers 1 PO1 HL 57278-02, 2 RO2 HL 41250-08A1, and 5RO1 AA11094. The government has certain rights in this invention.

STATEMENT REGARDING SEQUENCE LISTING

[0002] Applicants assert that the attached paper copy of the Sequence Listing for this utility application, a Continuation-in-Part (C.I.P.) to U.S. Patent Application No. 09/618,623, filed July 18, 2000 pending, is identical to the Sequence Listing in computer readable form found on the accompanying computer disk, as required by 37 CFR 1.821 (c) and is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] This invention relates to phospholipases and, more particularly, to novel calcium-independent phospholipase $A_2\gamma$ polypeptides and to nucleic acids encoding these polypeptides, as well as to methods of making and using these nucleic acids and polypeptides.

BACKGROUND OF THE INVENTION

[0004] The function of complex living biological organisms relies on the meticulous control of cellular activity, including close regulation of cell growth, proliferation and function. The family of enzymes known as the phospholipases A_2 has been implicated in the control of cellular activity by catalyzing the esterolytic cleavage of fatty acids from phospholipids, thereby regulating the release of lipid second messengers, cellular growth factors, and the properties of the cellular membrane (Samuelsson et al., Annu. Rev. Biochem. 47:997-1029, 1978; Moolenaar, W.H., Curr. Opin. Cell. Biol. 7:203-10, 1995). In particular,

by controlling the production of second messengers such as arachidonic acid and its biologically active eicosanoid metabolites, phospholipases A_2 are involved in modulating such processes as cellular growth programs, inflammation, vascular tone and ion channel function. (Needleman et al., Annu. Rev. Biochem. 55:69-102, 1986).

[0005] However, phospholipases A_2 are a broad family of enzymes with varying kinetic and physical properties, and distinct functions. Early research focused on distinguishing broad classes of the enzymes within the larger family. Several classes were distinguished using in vitro activity assays, and are categorized based on the dependence of their enzymatic activity on the presence of calcium ion. (See e.g., Demel et al, Biochim. Biopliys. Acta 406:97-107,1975). Thus, one class, the secretory phospholipases A_2 are distinguished by an obligatory dependence on high (millimolar) concentrations of calcium ions, as well as low molecular weights (14-18 kDa) and relative heat stability. (Demel et al., supra; Tischfield, J.A., J. Biol. Chem. 272:17247-50, 1997). The activity of a second class, the calcium-facilitated phospholipases A_2 is facilitated by the presence of nanomolar concentrations of calcium ions, but the presence of the calcium ion is not obligatory. (Loeb et al., J. Biol. Chem. 261:10467-70, 1986; Kramer et al., Biochim. Biophys. Acta 878:394-403; Glover et al., J. Biol. Chem. 270:15359-67, 1986). A third class of enzymes is entirely calcium-independent in vitro studies, and is also distinguished by a finely tuned inhibition by (E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL). (Wolf et al., J. Biol. Chem. 260:7295-303; Hirashima et al., J. Neurochem. 59:708-14; Lehman et al., J. Biol. Chem. 268:20713-16).

[0006] Application of molecular biological techniques has provided some insights into the structure and function of founding members in each class of phospholipases A_2 and has provided a further basis for distinguishing among the classes. (See, e.g. Demel et al, supra; Evenberg et al., J. Biol. Chem. 252: 1189-96, 1977; Tischfeld, J.A., J. Biol. Chem. 272: 17247-50, 1997). For example, members of the secretory phospholipases A_2 use a calcium ion to polarize the carbonyl for attack by a histidine-activated H_2O molecule, while the intracellular phospholipases use a nucleophilic serine. The calcium-facilitated phospholipases A_2 have a GXSGS consensus lipase motif (SEQ ID NO: 49), in contrast to the $iPLA_2$ group which has a GXSTG consensus motif (SEQ ID NO: 50). The calcium-independent phospholipases A_2 are also distinguished by a consensus sequence for nucleotide binding. (Andrews et al., Biochem. J. 252:199-206, 1988; Tang et al., J. Biol. Chem.

272:8567-75, 1998). These findings have clearly boosted progress toward identifying the polypeptides responsible for catalyzing the synthesis of the eicosanoid metabolites and toward understanding the regulatory mechanisms of phospholipases A_2 that are involved in normal and disease states.

[0007] The more recent developments of intense genome sequencing efforts have produced partial sequence data on the phospholipases and led to related structural insights. For example, two new calcium-facilitated phospholipases have recently been described based on data from protein and nucleotide databases. (Underwood et al., J. Biol. Chem. 273: 21926-32, 1998; Pickardetal., J Biol. Chem. 274: 8823-31, 1999). Further, during sequencing of the long arm of chromosome 7 in the Human Genome Sequencing Project, a predicted protein product of 40kDa was identified. The polypeptide contained two 10 amino acid segments homologous to the lipase and nucleotide-binding consensus sequences described for the founding members of the $iPLA_2$ family. (Tang et al., supra).

[0008] Earlier work has suggested a connection between some types of phospholipases and certain disease conditions in animals. For example, intensive study of reperfusion injury in myocardial tissue has led to the hypothesis that pathology is ultimately generated because of membrane phospholipid breakdown attributable to activation of myocardial phospholipase A_2 activity. (See e.g. Van der Vusse et al., Hydrolysis of phospholipids and cellular integrity, In: H.M. Piper (ed.) Pathophysiology of Severe Ischemic Myocardial injury, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1990, 167-93). Furthermore, calcium-dependent and calcium-independent phospholipase A_2 activities have also been found to be present in the human cerebral cortex. Some reports have suggested a possible link between the activity of both calcium-dependent calcium independent phospholipases A_2 and cortical degenerative diseases such as Alzheimer's disease. (For reviews, see e.g. Farooqui et al., Neurochem. Int. 30: 5 17-22, 1997; Farooqui et al., Brain Res. Bull. 49: 139-53, 1999).

[0009] Certain inhibitors of phospholipases A_2 have been identified as possible therapeutic candidates for treating PLA_2-mediated diseases. For example, fatty acid trifluoromethyl ketones, bromoenol lactone, methyl arachidonyl fluorophosphonate, bezenesulfonamides and other specific inhibitors of phospholipases A_2 have been shown to decrease PLA_2 activity and all have been considered for treating inflammatory diseases thought to be mediated by PLA_2. (See e.g. Farooqui et al, 1999, supra). Nevertheless, as

noted above, the phospholipases A_2, as well as the $iPLA_2$ subfamily itself, are a heterogeneous group of enzymes, with differing molecular weights, substrates, and responses to inhibitors. Because of this, the development of agents for treating diseases mediated by these compounds is ideally based upon determining and characterizing the structure and functional characteristics of the particular $iPLA_2$ involved in the disease process. Thus, it is important to identify and characterize the phospholipases A_2 family members of interest in order to identify and develop new drugs with specific affinity and/or activity for those phospholipases A_2.

BRIEF SUMMARY OF THE INVENTION

[00010] Accordingly, the inventors have discovered a novel phospholipase A_2, referred to herein as calcium-independent phospholipase $A_2\gamma$ ($iPLA_2\gamma$), and nucleic acid sequences encoding and expressing $iPLA_2\gamma$. The novel calcium-independent phospholipase $A_2\gamma$ is involved with supplying fatty acids for β-oxidation or hydrolyzing lipids to provide signaling molecules which regulate energy storage and bioenergenetics and is believed to be involved in certain disease processes in animals. More specifically, the present invention is based on the collective work reported herein which identifies the complete genomic organization, nucleic acid sequence, and lipase activity of the novel calcium-independent membrane-associated phospholipase A_2.

[00011] In one embodiment, the invention is directed to an isolated nucleic acid molecule comprising a set of $iPLA_2\gamma$ polynucleotides. In one aspect of this embodiment, the $iPLA_2\gamma$ polynucleotides encode and express an $iPLA_2\gamma$ polypeptide. The $iPLA_2\gamma$ polypeptide catalyzes cleavage of fatty acids from the phospholipids by either sn-1 or sn-2 hydrolysis. Preferred sequences of the $iPLA_2\gamma$ polypeptide are as set forth in SEQ ID NO: 1 and SEQ ID NO: 2. SEQ ID NO: 1 represents the amino acid sequences corresponding to alternative splice variants of $iPLA_2\gamma$, identified herein as gamma 1 and gamma 2, respectively. The two splice variants involve splice sites 5' to the start codon so that both splice variants gamma 1 and 2 encode the same $iPLA_2\gamma$ polypeptide SEQ ID NO: 1. However, splice variant gamma 3, SEQ ID NO:5 utilizes a start codon 5' to that of splice variant gamma 1 and splice variant gamma 2 to encode a longer polypeptide SEQ ID NO:2.

[00012] SEQ ID NO:3 and SEQ ID NO:4 constitute the cDNA of the coding regions of splice variant gamma 1 and 2, respectively. SEQ ID NO:5 constitutes the cDNA

of the coding region of splice variant gamma 3. SEQ ID NO: 6 is the nucleotide sequence for the full length iPLA$_2\gamma$ c DNA.

[00013] The present invention is also directed to iPLA$_2\gamma$ polynucleotides and fragments thereof which specifically hybridize under stringent conditions to the complements of the coding regions of iPLA$_2\gamma$ polynucleotides set forth in SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, and their corresponding complements, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, respectively.

[00014] In another aspect of this embodiment, the present invention is directed to iPLA$_2\gamma$ polynucleotides comprising polynucleotides having at least about 90% identity with SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9; and, preferably, such polynucleotides comprising SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6, or SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9.

[00015] In another aspect of this embodiment, the present invention is also directed to vectors and cells comprising a polynucleotide encoding an iPLA$_2\gamma$ polypeptide. In addition, the present invention encompasses antisense compounds which specifically hybridize to SEQ ID NO: 3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO: 6.

[00016] The present invention is also directed to iPLA$_2\gamma$ polynucleotides (SEQ ID NOS 13, 16, 19, and 22) which encode full-length or truncated polypeptides (SEQ ID NOS 1, 15, 18, 21) through use of alternative ATG start sites. Each of these constructs is identical but truncated compared to full-length iPLA$_2\gamma$ (SEQ ID NO: 1) including sequence corresponding to the lipase active site encoding amino acids 481-485) of iPLA$_2\gamma$ and thus each potentially retains catalytic activity. However, it is expected that modifications such as removal of an N-terminal membrane binding domain through use of a downstream ATG start site (or use of an alternative promoter through alternative splicing) will have profound effects on the functional properties of the polypeptide. These changes may increase or decrease enzymatic activity or specificity or change the intracellular site at which the polypeptide's activity is exerted. In fact, the major thrust of this patent is to describe the diversity of this molecule (in terms of splicing variants, use of alternative promoters, and use of downstream ATG start sites) with the implication being that the functional properties of this polypeptide are highly regulated by these diverse modifications.

[00017] It is possible that the full-length nucleotide sequence (SEQ ID NO: 13) encoding the 88kDa polypeptide (SEQ ID NO: 1) may not represent the true endogenous form of iPLA$_2\gamma$ having significant biologically functional activity. When constructs containing the full length 88kDa form (SEQ ID NO: 13) are expressed in the baculovirus system and in transgenic mice, 74 and 63kDa polypeptides are expressed. These results may indicate that the true endogenous activity derives from these truncated forms. It is also equally possible (and likely) that different forms of iPLA$_2\gamma$ derived from differential splicing or use of alternative ATG start sites or other mechanisms) are expressed to different degrees under different biological conditions. For example, we show that canine iPLA$_2\gamma$ is expressed both as different sized isoforms and at different levels in aorta vs. liver (Figure 30). Likewise, functional regulation of iPLA$_2\gamma$ in normal and pathological conditions may also be regulated at transcriptional and translational levels by generation of a alternatively spliced forms, alternative promoter usage, and use of alternative ATG start sites as well as by other mechanisms.

[00018] In a particular aspect of this embodiment, the present invention is directed to a fragment of an iPLA$_2\gamma$ polynucleotide comprising an iPLA$_2\gamma$ repressor binding site. Preferably, the polynucleotide comprises SEQ ID NO:10, 5'-ATGATTTCACGTTTAGCTCAATTTAAGCCAAGTTCCCAAATTTTAAGAAAAGTAT CGGATAGTGGCTGGTTAAAACAGAAAAACATCAAACA-3' or its complement SEQ ID NO:31 or subparts thereof further comprising SEQ ID NO:32, 33, 34, 35 or their complementary oligonucleotide pairs including appropriate sticky ends for ligation (SEQ ID NOS 36, 37, 38, or 39).

[00019] In another embodiment, the present invention is directed to an isolated polypeptide comprising a phospholipase A$_2\gamma$. The isolated polypeptide catalyzes cleavage of fatty acids from the phospholipids. In one aspect of this embodiment, the polypeptide has at least about 90% identity with SEQ ID NO:1 or SEQ ID NO:2; and, more preferably, the polypeptide comprises SEQ ID NO:1 or SEQ ID NO:2.

[00020] In another aspect of this embodiment, the present invention comprises a conservatively substituted variant of SEQ ID NO:1 or SEQ ID NO:2. The present invention also encompasses antibodies capable of binding to a phospholipase A$_2\gamma$ polypeptide.

[00021] Another embodiment of the present invention constitutes a method of treating inflammation in a patient. The method comprises treating the patient in need of such treatment to alter (decrease or increase) calcium-independent phospholipase $A_2\gamma$ activity in the patient. It is contemplated that patients in need of such treatment include those patients suffering from one or more of Alzheimer's disease, myocardial ischemia and myocardial infarction. Preferably, one method comprises administering to the patient an effective amount of phospholipase $A_2\gamma$ translational repressor molecule.

[00022] As used herein, the term "patient" includes humans and other living animals.

[00023] In another aspect of this invention, the method comprises administering to the patient an antisense sequence which specifically hybridizes to SEQ ID NO:3 or SEQ ID NO:4 or SEQ ID NO:5.

[00024] In another embodiment, the present invention is directed to a method of modulating fatty acid utilization in a living patient in need thereof. In this aspect, the method comprises increasing or decreasing $iPLA_2\gamma$ activity in the patient. Patients in need of such treatment include those patients suffering from diabetes and/or obesity. In an aspect, this method comprises administering to the patient a substance in an effective amount which blocks translational repression of $iPLA_2\gamma$, which results in increasing $iPLA_2\gamma$ activity in the patient. In another aspect of this invention, the method comprises administering to the patient an $iPLA_2\gamma$ polypeptide or a polynucleotide encoding said $iPLA_2\gamma$ polypeptide.

[00025] Another embodiment of the present invention is directed to a method for measuring activity of a phospholipase $A_2\gamma$ polypeptide of cells in a biological sample. The method comprises introducing into the sample a phospholipid substrate for cleavage of fatty acids by the phospholipase $A_2\gamma$, wherein the phospholipase $A_2\gamma$ cleaves fatty acid from either the sn-1 or sn-2-position of the phospholipid substrate. This method further comprises measuring cleavage of the phospholipid substrate and/or quantifying the release of fatty acid from the substrate.

[00026] The present invention is also directed to an assay method for identifying substances which modulate $iPLA_2\gamma$ expression in a cell. The method comprises effectively contacting a candidate substance with cells comprising a promoter sequence operably linked to an $iPLA_2\gamma$ repressor or enhancer binding site and a reporter gene and

measuring expression of the reporter gene. Alternatively, the method comprises contacting a candidate substance with cells comprising an $iPLA_2\gamma$ promoter sequence operably linked to a reporter gene and measuring expression of the reporter gene. By $iPLA_2\gamma$ repressor or enhancer binding site it is meant that portion of the nucleotide sequence of an $iPLA_2\gamma$ polynucleotide to which one or more endogenous substances within a cell binds to regulate transcription or translation of that $iPLA_2\gamma$ polynucleotide.

[00027] In one embodiment, the $iPLA_2\gamma$ repressor binding site comprises SEQ ID NO:10 or fragments SEQ ID NOS 32, 33, 34 and 35 or their complementary oligonucleotide pairs including appropriate sticky ends for ligation (SEQ ID NOS 36-39).

[00028] In another embodiment, the $iPLA_2\gamma$ promoter comprises sequences (generally within 2kb) upstream from $iPLA_2\gamma$ exons 1 or 2. The reporter gene preferably encodes an enzyme capable of being detected by a suitable colorimetric, fluorometric or luminometric assay such as, for example, a reporter sequence encoding and expressing a luciferase. In a particularly preferred embodiment, the promoter sequence is a baculovirus promoter sequence and the cells are Sf9 cells.

[00029] The present invention also encompasses genetically engineered cells capable of identifying substances which modulate $iPLA_2\gamma$ expression in a cell. Such cells comprise a promoter operably linked to an $iPLA_2\gamma$ repressor binding site and a reporter gene, or alternatively an $iPLA_2\gamma$ promoter linked to a reporter gene. Preferably, the $iPLA_2\gamma$ repressor binding site comprises SEQ ID NO:10 while the $iPLA_2\gamma$ promoter comprises a sequence within 2kb upstream of exons 1 or 2. This reporter gene preferably encodes an enzyme capable of being detected by a suitable colorimetric, fluorometric or luminometric assay such as, for example, a reporter sequence encoding a luciferase. In an aspect, the promoter sequence is a baculovirus promoter sequence and the cells are Sf9 cells.

[00030] The present invention includes a method for identifying substances which modulate $iPLA_2\gamma$ expression. In an aspect, the method comprises contacting a candidate substance with cells capable of expressing $iPLA_2\gamma$ or a fragment thereof, and measuring the expression of $iPLA_2\gamma$ or a fragment thereof by the cells. A level of expression greater or less than expression in an absence of the substance indicates and is determinant of activity in modulating $iPLA_2\gamma$ expression.

[00031] Therefore, the present invention affords several advantages, including the provision of a novel phospholipase $A_2\gamma$, $iPLA_2\gamma$, which catalyzes the release of fatty acids from the sn-1 or sn-2-position of phospholipid substrate; the provision of methods for identifying a substance which represses or enhances and thus controls $iPLA_2\gamma$ expression in a sample; the provision of methods for treating diseases involving inflammatory processes, inflammation or fatty acid utilization; and the provision of methods for determining the presence and level of activity or expression of $iPLA_2\gamma$ in a sample.

[00032] In one embodiment, a transcriptional repressor region was defined including nucleotide residues 1-315 (SEQ ID NO: 57) of the 88 kDa coding sequence (SEQ ID NO: 13) utilizing gel shift analysis.

[00033] In another embodiment, a human $iPLA_2\gamma$ N-terminal mitochondrial localization signal and cleavage site are defined comprising sequence MISRLAQFKPSSQILRKΔVS (SEQ ID NO: 58). Cleavage occurs after the lysine (K) in $iPLA_2\gamma$.

[00034] In one embodiment, a transcription factor binding region is defined between nucleotide residues 6-50 encoding the 88 kDa protein and including the sequence 5'-TATTAATCTGACTGTAGATATATATATTTACCTCCTTAGTAATGC –3 (SEQ ID NO: 59) within the N-terminal coding region of $iPLA_2\gamma$ by gel shift analysis.

[00035] In another embodiment, three MyoD transcription factor binding sites (E-boxes) defined by the consensus nucleotide sequence CANNTG are identified in promoter 1 (pre exon 1) sequence of the $iPLA_2\gamma$ gene. The MyoD binding sites are located at nucleotide residues –22 through –27 corresponding to nucleotide sequence 5'-CAAGTG-3' (SEQ ID NO: 60), -53 through –58 corresponding to nucleotide sequence 5'-CAGGTG-3' (SEQ ID NO: 61), and –349 through –354 corresponding to nucleotide sequence 5'-CAGGTG-3' (SEQ ID NO:62) upstream from start of exon 1(see SEQ ID NO: 29 in Table I).

[00036] In another embodiment, utilizing gel shift analysis, we provide evidence for an initiator (Inr) sequence with a consensus sequence of Py-Py-A-N-T/A-Py-Py at which nuclear protein constituents bind to 5'- GCG TCA CTT CCG CTG GGG GCG G-3' (SEQ ID NO: 77) at nucleotide residues –54 through –75 upstream from the putative start of exon 2 (Figure 39). The Inr as well another sequence 5'-GCCAGTGTTTG-3' (SEQ ID NO

78) which is consistent with sequence for a CORE promoter element are highly conserved in comparisons of human, mouse, and rat sequence within this region of the $iPLA_2\gamma$ gene (see Figure 39).

BRIEF DESCRIPTION OF THE DRAWINGS

[00037] Figure 1 shows the $iPLA_2\gamma$ cDNA sequence (SEQ ID NO:6) which contains the coding region for the 88kDa $iPLA_2\gamma$ polypeptide along with the corresponding amino acid sequence (SEQ ID NO:1).

[00038] Figure 2 illustrates three alternative splice variants of the $iPLA_2\gamma$ gene.

[00039] Figure 3 illustrates the 5' portion of three alternative splice variants, identified as 1 (SEQ ID NO:3), 2 (SEQ ID NO:4) and 3 (SEQ ID NO:5) along with amino terminal portion of the encoded polypeptides corresponding to splice variants 1 and 2 (SEQ ID NO:1) and splice variant 3 (SEQ ID NO:2).

[00040] Figure 4 illustrates the full-length 88kDa polypeptide (SEQ ID NO:1) and the cDNA encoding the polypeptide (SEQ ID NO:13) along with sense primer M444 (SEQ ID NO:11) and reverse primer M458 (SEQ ID NO:12) used in the PCR (polymerase chain reaction) amplification of the 88kDa polypeptide.

[00041] Figure 5 illustrates the 77kDa truncated polypeptide starting at amino acid 101 and nucleotide 301 (SEQ ID NO:15) and the cDNA encoding the polypeptide (SEQ ID NO: 16) along with sense primer m534 (SEQ ID NO: 14) and reverse primer M458 (SEQ ID NO:12) used in the PCR amplification of the 74kDa polypeptide.

[00042] Figure 6 illustrates the 74kDa truncated polypeptide starting at amino acid 122 and nucleotide 364 (SEQ ID NO:18) and the cDNA encoding the polypeptide (SEQ ID NO:19) along with sense primer m533 (SEQ ID NO:17) and reverse primer M458 (SEQ ID NO:12) used in the PCR amplification of the 74kDa polypeptide.

[00043] Figure 7 illustrates the 63kDa truncated polypeptide starting at amino acid 221 and nucleotide 661 (SEQ ID NO:21) and the cDNA encoding the polypeptide (SEQ ID NO:22) along with sense primer m530 (SEQ ID NO:20) and reverse primer M458 (SEQ ID NO:12) used in the PCR amplification of the 74kDa polypeptide.

[00044] Figure 8 is a schematic illustration mapping expressed sequence tags (ESTs) overlapping the full-length sequence of $iPLA_2\gamma$.

[00045] Figure 9 illustrates the analysis of PCR amplified human $iPLA_2\gamma$ on agarose gel electrophoresis.

[00046] Figure 10 shows a potential alternative exon 5 splice variant of human $iPLA_2\gamma$. In gene splicing, introns typically begin with the dinucleotide "gt" and end with "ag". This gt/ag splice signal occurs with a frequency of 98.71% in gene splicing and can be used as a reliable tool for identifying the boundaries of exons in genes. The previously reported 3' splice signal of $iPLA_2\gamma$ exon 5 is illustrated in 'A'. This splice site utilizes an unusual gc/ag splice signal which is utilized at a frequency of only 0.56% in splicing of genes. The use of the more common gt/ag splice signal will result in splicing shown in 'B'. As illustrated, this splicing will not alter the reading frame of the encoded protein but will result in a modified portion of the $iPLA_2\gamma$ sequence which contains the sequence "ASCSV" (SEQ ID NO: 28) in place of "IIAR" (SEQ ID NO: 51) shown in 'A'. Sequence encoding the altered sequence in 'B' has not yet been cloned from a cDNA library nor has sequence with identity to SEQ ID NO: 28 been reported in GenBank® (NIH genetic sequence database). However, because of the extensive alternative splicing that the $iPLA_2\gamma$ gene undergoes (at least 10 splice variants), it is at least plausible that the more common gt/ag splicing may be utilized at the 3' end of exon 5 resulting in a polypeptide containing SEQ ID NO: 28. Because of the proximity of the amino acids affected by this alternative splicing to the lipase consensus site (within 126 amino acids), the generation of a polypeptide with SEQ ID NO: 28 could potentially result in modulation of $iPLA_2\gamma$ activity or functional properties.

[00047] Figure 11 is a hydropathy plot analysis of the deduced amino acid sequence of human $iPLA_2\gamma$.

[00048] Figure 12 is a Northern blot analysis of human $iPLA_2\gamma$ mRNA obtained from multiple tissue samples.

[00049] Figure 13 is a schematic illustration of the genomic organization of the human $iPLA_2\gamma$ gene.

[00050] Figure 14 shows the results of gel electrophoresis and autoradiographic visualization of the in vitro translation products of $iPLA_2\gamma$.

[00051] Figure 15 shows the results of Western blot analysis of $iPLA_2\gamma$ expression in Sf9 cells.

[00052] Figure 16 is a graph of an initial rate analysis of the $iPLA_2$ activity of $iPLA_2\gamma$.

[00053] Figure 17 is a substrate selectivity profile of the $iPLA_2$ activity of $iPLA_2\gamma$.

[00054] Figure 18 is a graph of inhibition of $iPLA_2$ activity of $iPLA_2\gamma$ by BEL.

[00055] Figure 19 shows the results of Western analysis of expression of truncated $iPLA_2\gamma$ constructs in Sf9 cells.

[00056] Western analysis is sometimes called Western blotting or immunoblotting and is a reliable method for checking any sample for the presence of a specific antigen. The detection is based on the molecular weight of an antigen and the interaction of the antigen with a specific primary antibody.

[00057] Figure 20 is a schematic representation of $iPLA_2\gamma$ translational repression constructs.

[00058] Figure 21 is a schematic representation of the pFASTBAC vector containing gamma 23mer sequences and luciferase coding sequence.

[00059] Figure 22 is a schematic representation of baculovirus promoter constructs containing gamma 23mer sequences and luciferase coding sequence.

[00060] Figure 23 shows phosphorylated oligo pairs for sequences between nucleotide 364 and nucleotide 455 for use in translational repression of $iPLA_2\gamma$ in the luciferase expression system.

[00061] Figure 24 is a bar graph providing luciferase assay results showing the relative levels of expression of three different $iPLA_2\gamma$ constructs in Sf9 cells.

[00062] Figure 25 shows the N-terminal regions of mouse (SEQ ID NO: 40), rat (SEQ ID NO: 41) and human $iPLA_2\gamma$ (SEQ ID NO: 42) aligned using the Internet-based MultAlign program. MultAlign does a simultaneous alignment for two or more DNA or

protein sequences. The program is based on a generalization of the algorithm of M.S.Waterman, T.F.Smith and W.A.Beyer (Adv.Math. Vol. 20, pp. 367- 387 (1976)) by M.Krueger and G. Osterburg (Comp. Prog. in Biomed. Vol. 16, pp. 68- 69 (1983)).

[00063] Regarding Figure 25, arrows indicate methionine residues at which the 88kDa, 77kDa, 74kDa, and 63kDa polypeptides are encoded for the human iPLA$_2\gamma$ protein. Boxes shown in Figure 25 indicate regions of 100% conservation of amino acid sequence across all three species.

[00064] Further, regarding Figure 25, overall, the N-terminal sequence of iPLA$_2\gamma$ is highly conserved across all three species (71%). Methionine residues corresponding to the 88kDa , 77kDa, 74kDa and 63kDa polypeptides are conserved across all three species. The conservation of these methionine residues may suggest their use in the mouse and rat for downstream initiation of truncated forms of iPLA$_2\gamma$ as found in the human. Interestingly, mouse and rat sequences have an additional methionine residue, not present in human iPLA$_2\gamma$, upstream from the 77kDa start site which may potentially also be used for alternative initiation and synthesis of truncated iPLA$_2\gamma$ forms.

[00065] Figure 26 shows electrospray ionization mass spectroscopy of phospholipids in wild-type and transgenic myocardium. FIG 26(a) shows a comparison of phosphatidylcholine, ethanolamine glycerophospholipid, plasmalogen, and triacylglyceride levels in fed (open bars) and 16h fasted (closed bars) WT mice. $P < 0.01$ ($n = 3$). FIG 26(b) shows a comparison of phosphatidylcholine, ethanolamine glycerophospholipid, plasmalogen, and triacylglyceride levels in fed (open bars) and 16h fasted (closed bars) transgenic mice. $P < 0.01$. In (a) and (b), other molecular species representing <2% of the total pools were also identified without demonstrable differences between control and transgenic mice.

[00066] Figure 27 shows the result of Northern blot analysis of mRNA extracted from Sf9 cells infected for 48 h with recombinant iPLA2γ baculoviral constructs and then treated with actinomycin D for 0, 0.25, 0.5, 1.0, 2.0 and 4 hours (to resolve RNA stability). 88kDa construct contained full length iPLA2γ coding sequence (SEQ ID NO: 6) encoding the 88kDa protein (SEQ ID NO: 1). The 74kDa construct contained truncated sequence (SEQ ID NO: 19) encoding the 74kDa protein (SEQ ID NO: 18). The 63kDa

construct contained truncated sequence (SEQ ID NO: 22) encoding the 63kDa protein (SEQ ID NO: 21).

[00067] Figure 28 shows the observations from quantitative PCR analysis of RNA stability of truncated iPLA$_2\gamma$ expression in Sf9 cells.

[00068] Figure 29 shows expression of truncated iPLA$_2\gamma$ forms in an in vitro expression system. Truncated iPLA$_2\gamma$ constructs in vector pEF were expressed using a TnT® Quick Coupled Transcription Translation System and analyzed by SDS PAGE. The TnT® Quick Coupled Transcription/Translation Systems are single-tube, coupled transcription/translation reactions for eukaryotic in vitro translation. 88kDa, 77kDa, 74kDa, 70kDa, and 63kDa truncated forms are represented as 88, 77, 74, 70, and 63. Molecular weight standards are shown on the left and the predicted corresponding sizes of each of the iPLA2γ isoforms synthesized from alternative initiator methionines are indicated on the right.

[00069] Figure 30 shows the results of a Western blot analysis of extracts isolated from canine aorta homogenate and liver peroxisomes along with cellular extract from Sf9 cells infected with a full-length iPLA$_2\gamma$ construct. Western blotting, an extension of protein electrophoresis, is sometimes called immunoblotting and is a reliable method for checking any sample for the presence of a specific antigen. The detection is based on the molecular weight of an antigen and the interaction of the antigen with a specific primary antibody. Samples are prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation. The complex protein mixture from the homogenized sample is separated by size using SDS-PAGE and then transferred to a membrane for detection with a primary antibody.

[00070] Regarding, Figure 30, canine aorta and liver express different molecular weight forms of iPLA$_2\gamma$. Furthermore the mass of the canine aorta form is approximately 5 fold greater than the liver form. The lower band in the canine aorta lane is an artifact of the Western staining procedure since it is present even in the absence of primary (anti- iPLA$_2\gamma$) antibody. The upper band in the aorta lane is approximately 60kD while the liver form is approximately 66kD. While the expressed forms do not correspond in mass to the expressed forms from the baculoviral expression system, the significance of these results is three fold: 1) These results represent the first reported detection of endogenously expressed iPLA$_2$; 2) These results demonstrate that iPLA$_2\gamma$ is differentially processed to different

molecular masses in different tissues; and 3) These results demonstrate that in the endogenous state, the iPLA$_2\gamma$ isoforms are expressed to different degrees in different tissues. The significance of the latter two findings is that these results show, that iPLA$_2\gamma$ is a molecule which undergoes regulated expression (resulting in truncated forms and differential expression). Several mechanisms are disclosed herein by which this regulation can occur (alternative splicing, alternative promoter usage, and use of alternative initiator methionines). Regulation by these means has been demonstrated in artificial expression systems (baculoviral expression or transfection of cells with truncated constructs). These results clearly show that a similar regulation also occurs endogenously. Undoubtedly, additional means of regulation are also used endogenously, e.g. phosphorylation, myristylation, or proteolysis.

[00071] Figure 31 shows a diagrammatic representation of the functional domains of iPLA$_2\gamma$. The dark rectangle represents the full-length iPLA$_2\gamma$ protein (SEQ ID NO: 1). The locations of alternative initiator methionine residues within the region "alternative start sites" and encoding 88kDa, 77kDa, 74kDa, and 63kDa polypeptides are indicated as 88, 77, 74, and 63 respectively. The N-terminus of the protein is also identified as the membrane binding region due to the hydrophobicity of this region of the polypeptide. Within the "alternative start site" region is the putative "inhibitory region" C-terminal of the 74kDa start site. The ATP binding motif, lipase consensus site, and peroxisomal localization signal site within the "patatin homologous region" are also identified. Amino acid number is indicated by the scale at the top. Sequences are shown in SEQ ID NOS 95-103, left to right, respectively, starting from the top.

[00072] A predicted mitochondrial import signal region is shaded and the putative cleavage site (LRK/VS) (SEQ ID NO: 95) is indicated. This import signal and cleavage site were predicted by the internet program Mitoprot (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter). In a preliminary study we have shown that in vitro synthesized product corresponding to the 74 kDa protein is cleaved to a smaller protein (approximately 72 kDa) in a mitochondrial uptake assay. These results lend support to our prediction that the 74 kDa iPLA$_2\gamma$ has a mitochondrial import signal which is cleaved during import into the mitochondria. Based on this information, iPLA$_2\gamma$, appears to be a protein which has both a peroxisomal as well as a mitochondrial localization. The regulation of the intracellular localization of iPLA$_2\gamma$ has important

implications in regard to its potential role in modulation of cellular functions. For example, a peroxisomal localization may imply utilization of lipids for heat generation in uncoupling ad free radical generation while a mitochondrial localization may suggest that lipids released through $iPLA_2\gamma$ action are utilized for cellular energy metabolism and function in the cell or coupling of mitochondrial oxidation and thermogenesis by uncoupling in the mitochondrial membrane. It should also be specifically pointed out that the actions of $iPLA_2\gamma$ on membrane lipids likely affects the function of membrane associated proteins such as ion channels which can dramatically affect cellular function. Other computer predicted sites of interest are also indicated including a zinc finger, Kringle site, SAP putative DNA binding motif, membrane retention signal, amidation site, and putative M-protein repeats. These significance of these predicted domains remains to be elucidated by further studies including expression of site directed mutated forms of $iPLA_2\gamma$.

[00073] Figure 32 shows a total of twelve potential splice variants of $iPLA_2\gamma$. A representation of the gene sequence is shown in the middle of the diagram with exons 1-13 (indicated as boxes) numbered in the diagram. Splice variants 1-XII are represented by the exons they contain (boxes) connected by lines which represent the splicing out of intron sequences. For example, splice variant I contains exon 2, a truncated exon 5 (T5), and exons 6, 7, 8, 9, and 10. A line extends beyond exon 10 to indicate the presence or absence of exons 11-13 are unknown at the present. Incomplete sequence is available for variants I, II, III, V, VIII, X, and XI, In contrast, the full sequence of splice variant IV is known and it contains exons 2,5,6,7,8,9,10,11,12, and 13.

[00074] These results suggest that two basic types of splice variants of $iPLA_2\gamma$ exist, those initiating from exon 1 and those initiating from exon 2. Thus far, no splice variants have been identified containing both exons 1 and 2 either through analysis of sequences within GenBank® or by RTPCR analysis performed in our laboratory. These results suggest that $iPLA_2\gamma$ utilizes two different promoters upstream of exons 1 and 2 respectively. That many splice forms of $iPLA_2\gamma$ have been identified supports our belief that endogenous synthesis of $iPLA_2\gamma$ is a highly regulated process.

[00075] Figure 33 shows the analysis of promoter activity of the $iPLA_2\gamma$ nucleotide sequence upstream from exon 2.

[00076] Figure 34 shows promoter activity of the pre exon 1 and 2 regions. Sequence corresponding to approximately 600 nt upstream of exons 1 and 2 were PCR amplified from the genomic BAC clone AC005058 and cloned upstream of the promoterless vector, pGL3E (Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711) to drive expression of the luciferase gene when transfected into CV1 cells. Pre exon 2 sequence had high promoter activity compared to the pGL3P control vector containing SV40 promoter. In contrast, the pre exon 1 sequence had negligible promoter activity as did the pGL3E promoterless negative control vector. These results suggest that pre exon 2 sequence is used endogenously for expression of $iPLA_2\gamma$ initiating from exon 2. The presence of sequences initiating from exon 1 suggests that sequences upstream from exon 1 are an alternative promoter region for $iPLA_2\gamma$ expression. These results provide further evidence that the $iPLA_2\gamma$ expression is regulated by alternative promoter usage. It is easy to speculate that pre exon 1 promoter sequence may be required under specific cell conditions such as differentiation or under conditions of stress. The lack of demonstrable promoter activity from pre exon 1 sequence may suggest that additional specific transcription factors may be required to initiate transcription in the CV1 system or may indicate that additional upstream sequences are required for promoter activity.

[00077] Figure 35 shows the homologies between the three members of the patatin family of proteins: potato patatin (alpha), the $iPLA_2\beta$ protein described in U.S. Patent No. 5,976,854 which issued to Simon Jones, et al. on November 2, 1999 (beta), and the iPLA2γ protein described and claimed in this application (gamma). Also shown are representations of two C. elegans protein sequences reported in GenBank® which are $iPLA_2\gamma$ homologs. All the proteins represented contain a common region of homology to patatin (stippled box). Within this region are ATP and lipase consensus sites as indicated by the shaded boxes. That these proteins share a patatin domain has important implications with regard to their evolution, strongly implying that all were evolved from a common ancestor related to potato patatin and eventually diverged into three separate classes of $iPLA_2$ molecule, alpha, beta, and gamma.

[00078] In addition to the patatin domain, iPLA2β (beta) has eight ankyrin repeats and a calmodulin binding domain. $iPLA_2\gamma$ has 4 alternative ATG start sites at which proteins of 88, 77, 74, and 63kDa can be synthesized. $iPLA_2\gamma$ also has a C-terminal SKL peroxisomal localization sequence. To further distinguish $iPLA_2\beta$ from $iPLA_2\gamma$, it should

further be pointed out that: 1) They do not share any significant homology in their N-terminal halves; 2) They are encoded by separate genes on different chromosomes (iPLA$_2\gamma$ is on 7 while iPLA$_2\beta$ is on 22); 3) Their respective genes contain different numbers of exons (13 for iPLA$_2\gamma$ and 16 for iPLA$_2$ β); 4) That, unlike iPLA$_2\beta$, iPLA$_2\gamma$ has two alternative promoters; and 5) iPLA$_2\beta$ and iPLA$_2\gamma$ have different intracellular localizations (membrane associated and peroxisomal for iPLA$_2\gamma$ vs. mitochondrial and primarily cytosolic for iPLA$_2\beta$). Like iPLA$_2\gamma$, the C. elegans proteins contain the patatin region of homology with ATP and lipase consensus sites and they do not contain ankyrin repeats. Blast analysis indicates that, in terms of amino acid homology, these C. elegans sequences are most like iPLA$_2\gamma$ and thus are likely to represent the C. elegans homolog of human iPLA$_2\gamma$and belong to the class of molecules designated 'gamma' or iPLA$_2\gamma$.

[00079] The identification of any new or otherwise unknown sequence from sequencing databases as being an iPLA$_2\gamma$ is made by performing NCBI Blast analysis (http://www.ncbi.nlm.nih.gov/BLAST/) of the sequence in question. Identity or a high degree of alignment with our reported GenBank® sequence (accession number AF263613 or with SEQ ID NOS 3, 4, 5, 6, 13, 16, 19, 22) will characterize the sequence as iPLA$_2\gamma$ or a homolog thereof. Figure 25, for example shows the homology between mouse, rat, and human iPLA$_2\gamma$. These sequences are all found in the GenBank® database. Boxed areas show amino acids which are identical between the three species.

[00080] Figure 25 reveals the high degree of N-terminal similarity of iPLA$_2\gamma$ from these three species. Within the first 210 amino acids, 145 amino acids are precisely conserved (69%). Comparing the full-length mouse iPLA$_2\gamma$ sequence (GenBank® accession number NP_080440), there is 82% identity of amino acid sequences when compared with the human sequence (SEQ ID NO: 1). If conservation of amino acid type is included, the homology rises to 90%. The differences in the sequences represent species differences. Nonetheless, all three sequences are clearly iPLA$_2\gamma$ and likely have the same or very similar functional attributes. In contrast, a similar comparison reveals only 38% homology of mouse NP_080440 to human iPLA$_2\beta$.

[00081] Other species specific homologs of iPLA$_2\gamma$ exist with lower homology. The C. elegans polypeptide, GenBank® accession number NP_500969 shares 57% homology with human iPLA$_2\gamma$ (SEQ ID NO: 1) while C. elegans polypeptide, GenBank® accession number AAF39894, shares 38% homology. And each is more homologous with iPLA$_2\gamma$ than

iPLA$_2\beta$ sequence. Furthermore, NP_500969 contains sequence which is an identical match at the human iPLA2γ lipase ICGVSTG (SEQ ID NO: 52) and ATP binding LSIDGGGTRG (SEQ ID NO: 53) domains (where underlined amino acids are also identically conserved in other polypeptides containing these domains). And so, in all likelihood, sequence NP_500969 represents the C. elegans homolog of human iPLA$_2\gamma$. Phylogenetic analysis suggests that a common ancestor gave rise to three main branches, the patatin, iPLA$_2\beta$ and iPLA$_2\gamma$ homologs. The iPLA$_2\gamma$ branch includes the highly homologous human, mouse, and rat sequences as well as less homologous sequences from C. elegans (AAG23484, CAA92615, T33857, AAK21368, and AAF39894) and Plasmodium falciparum (AAC71877). As of yet, only the human sequence has been expressed and has calcium independent phospholipase A_2 activity. However, each of these, with the exception of AAC71877 and AAF39894 contains closely linked regions with perfect matches to the lipase (GxSxG) and ATP binding (GxGxxG) consensus sequences suggesting that these sequences represent authentic iPLA$_2\gamma$ in other species.

[00082] Figure 36 illustrates the identification of a transcriptional regulatory domain within the 5' coding region of iPLA$_2\gamma$ using a luciferase reporter assay system.

[00083] Figure 37 illustrates gel mobility shift analysis (gel shift) of the iPLA$_2\gamma$ transcriptional regulatory domain within the 5' coding region of iPLA$_2\gamma$ for identification of a nuclear binding domain utilizing oligonucleotide CdxA (SEQ ID NO 59).

[00084] Figure 38 illustrates Immunoblot analysis of iPLA$_2\gamma$ in subcellular fractionations of rat heart showing differential expression of iPLA$_2\gamma$ isoforms in different subcellular fractions.

[00085] Figure 39 illustrates alignment of mouse (SEQ ID NO: 88), rat (SEQ ID NO: 89), and human (SEQ ID NO:90) pre-exon 2 sequence identifying an initiator (Inr) site as well as a putative core promoter element.

[00086] Figure 40 illustrates competitive gel retardation analysis of iPLA$_2\gamma$ regions identifying a transcription factor binding site Inr defined by SEQ ID NO: 62.

[00087] Figure 41 illustrates MyoD stimulation of promoter activity in pre-exon 1 sequence of iPLA$_2\gamma$ by measuring luciferase expression following transfection of CV1 cells with promoterless vector (pGLE) or with a construct in which pre exon 1 sequence

is inserted upstream of the luciferase gene in pGLE (pre-exon 1 with (+) or without (-) cotransfection with myoD vector. The full length DNA sequence is shown in SEQ ID NO: 84, while the E-box (MyoD) oligonucleotides are shown in SEQ ID NOS 85-87, respectively, in order of appearance.

[00088] Figure 42-A,B,C illustrates import of GFP into mitochondria mediated by an N-terminal iPLA$_2\gamma$ mitochondrial import signal in HeLa cells transfected singly or cotransfected with 74GFP and DSRed2-Mito constructs and visualized by confocal microscopy.

[00089] We demonstrate herein: 1) the presence of nuclear protein constituents which bind to the repressor region of the 5' region of iPLA$_2\gamma$ by gel shift analysis; 2) tissue specific translational regulation of iPLA$_2\gamma$; 3) the presence of a mitochondrial localization signal in the N- terminus of the major heart iPLA$_2\gamma$ isoforms. The presence of dual localization signals in discrete iPLA$_2\gamma$ isoforms regulated by tissue-specific genetic programs identifies a heretofore unanticipated complex interplay between cardiac mitochondria and peroxisomes for the uptake and utilization of the nascent iPLA$_2\gamma$ protein. Since the rate determining step in mitochondrial uncoupling protein (UCP) function is the interaction of mitochondrial non-esterified fatty acids with UCP, the results identify a diverse repertoire of iPLA$_2\gamma$ proteins which likely serve as important metabolic determinants integrating mitochondrial respiration and thermogenesis.

[00090] In summary, human iPLA2γ is synthesized from a single gene made up of 13 exons on chromosome 7q31. Naturally occurring polymorphisms are likely to result in a small percentage of nucleotide or amino acid substitutions (probably less than 1%). On the other hand, alternative splicing of the gene results in at least 10 different splice variants. Since the splicing occurs at the 5' end of the gene and there is no evidence that splicing involves the lipase active site, each of the splice variants potentially has phospholipase A2 activity. Alternative splicing is a well recognized mechanism of modulation of protein function (Leff, et al. Annual Rev Biochem 55: 1091-1117, 1986) and can affect many cellular processes such as signal transduction, transcriptional regulation, cellular transformation, and subcellular localization. A number of genetic defects have been associated with incorrect splicing. Alternative splicing of iPLA$_2\gamma$ may thus be a functional mechanism for regulating iPLA$_2\gamma$ activity in cells in terms of both the amount of activity as well as the specific

intracellular sites at which the activity occurs. Further modulation occurs in the form of truncation by use of downstream alternative ATG start sites resulting in 77kDa, 74kDa, and 63kDa polypeptides. This truncation is also likely to have important functional consequences. Indeed, we have shown that transgenic mice generated using a construct containing SEQ ID NO: 6 which encodes the 88kDa protein (SEQ ID NO: 1) express 74kDa and 63Da proteins (SEQ ID NOS 18 and 21). Additionally, we showed that canine iPLA2γ (which cross reacts with antibodies to human iPLA$_2$γ) is endogenously expressed as two different isoforms. Homologs of iPLA$_2$γ have been identified in other species besides canine including rat, mouse which share as much as 90% homology with human iPLA$_2$γ, and in C. elegans which share approximately 38-57% homology with human iPLA$_2$γ.

[00091] Three additional exons in the iPLA$_2$γ gene have been identified. For identification of these exon sequences, we used the same approach employed in our publication (Mancuso et al. J. Biol. Chem. 275: 9937-9945, 2000), alignment of raw genomic and coding (EST) sequences in GenBank® as well as comparison of these sequences with sequences cloned in our laboratory from 5' RACE of human cDNA libraries and identification of 5' and 3' splice signals.

[00092] These new exon sequences are shown in Table 1. It should be appreciated that these sequences were present in GenBank® as "raw" sequence within a much larger submitted block of sequence. Within this sequence, they were not identified as being iPLA$_2$γ sequence nor were they identified as exons or introns nor were the 5' and 3' boundaries noted, nor were these sequences identified as having any potential functional or otherwise significance other than being human genomic or coding sequence. We now identify and characterize these sequences as being exons within the iPLA$_2$γ gene and present detailed information in Table 1 including exon number, length of each exon, the 5' intronic sequence including the 'ag' splice signal for exon 4, nucleotide sequences of exon 1 and 4 (SEQ ID NOS: 29 and 30, respectively), and 3' intron sequence with the 'gt' splice signal bolded.

[00093] Table II shows that, in addition to the three splice variants described in Figure 2, we have identified an additional eight (8) iPLA2γ splice variants (for a total of eleven (11) splice variants) through examination of the GenBank® databases as well as by cloning and sequencing of RTPCR in our laboratory.

[00094] Table III illustrates PCR Primers (SEQ ID NO: 63-74) for analysis of $iPLA_2\gamma$ regulatory domain.

[00095] Table IV illustrates splice junctions of $iPLA_2\gamma$ splice variants III (SEQ ID NOS: 91-92) and X (SEQ ID NOS: 75-76).

[00096] Table V illustrates sequence surrounding $iPLA_2\gamma$ AUG start sites (SEQ ID NO: 79-82) in comparison with Kozak consensus sequence (SEQ ID NO: 83).

DETAILED DESCRIPTION OF THE INVENTION

[00097] The present invention is based upon our identification, isolation and sequencing (characterization) of the novel calcium-independent phospholipase A_2, $iPLA_2\gamma$. The new $iPLA_2\gamma$ is both membrane-associated and highly sensitive to inhibition by (E)-6-(bromomethylene)-3-(1 -napthalenyl)-2H-tetrahydropyran-2-one (BEL), which distinguishes our new $iPLA_2\gamma$ from previously reported membrane-associated phospholipases A_2. Prior to this invention, $iPLA_2\gamma$ was unknown and had not been distinguished as a biologically active substance discrete from known calcium-independent phospholipases A_2, nor had it been isolated in pure form and its putative sequences determined.

[00098] Reference to $iPLA_2\gamma$ or $iPLA_2\gamma$ polypeptides herein is intended to be construed to include the polypeptides corresponding to coding regions of three spliced variants identified herein. The amino acid sequences of the polypeptides are as set forth in SEQ ID NO:1 and SEQ ID NO:2. The coding portion of the cDNA of the splice variants are as set forth in SEQ ID NOS 3 and 4 (splice variants gamma 1 and gamma 2) and SEQ ID NO:5 (splice variant gamma 3) (see Figures 2-4). The $iPLA_2\gamma$ polypeptides within the present invention are also intended to include $iPLA_2\gamma$ of any origin which are substantially identical to and which are biologically equivalent to the $iPLA_2\gamma$ polypeptides characterized and described herein. Such substantially identical $iPLA_2\gamma$ may be native to any tissue or species and, similarly, biological activity can be characterized in any of a number of biological assay systems.

[00099] As used herein, the term "biologically equivalent" is intended to mean that the compositions of the present invention are capable of demonstrating some or all of the same sn-2-lipase activity, membrane localization and sensitivity to BEL inhibition, however,

not necessarily to the same degree as the iPLA2γ isolated herein as described in the examples below.

[000100] By the phrase "substantially identical" it is meant that the degree of identity of human $iPLA_2\gamma$ to the $iPLA_2\gamma$ from any species is greater than that between the $iPLA_2\gamma$ and any previously reported member of the $iPLA_2$ family. Sequence identity or percent identity is intended to mean the percentage of same residues between two sequences when the two sequences are aligned by methods well known to those skilled in the art (for example, see Higgins et al, Cabios 8:189-191, 1992; Dayhoff et al., in ATLAS OF PROTEIN SEQUENCE AND STRUCTURE, Dayhoff, Ed., NDRF, Washington, Vol. 5, suppl. 3, p. 345, 1978).

[000101] Further, polypeptides considered to be included within the term $iPLA_2\gamma$ polypeptides also includes conservatively substituted variants of SEQ ID NO:1 or SEQ ID NO:2. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is seine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups include: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine, glutamic acid-aspartic acid, leucine-methionine; glutamine-histidine.

[000102] In one embodiment, $iPLA_2\gamma$ encompasses a class of molecule which shares homology with human $iPLA_2\gamma$ (SEQ ID NO: 1), includes a patatin domain and ATP binding and lipase consensus sites, and is phylogenetically more closely related to $iPLA_2\gamma$ sequence than either of the other members of the patatin derived proteins, $iPLA_2\gamma$ or potato patatin. In this embodiment, $iPLA_2\gamma$ would include mouse and rat homologs sharing 90% homology as well as other homologs from other species including C. elegans or Plasmodium falciparum which, for example, share 38-57% homology with human $iPLA_2\gamma$ (SEQ ID NO: 1). In another embodiment, $iPLA_2\gamma$ includes sequences from species sharing approximately 90% homology with SEQ ID NO: 1 and in particular human sequences derived from the

iPLA$_2\gamma$ gene on chromosome 7 which include at least 11 splice variants (Table 2) as well as truncated polypeptides derived from use of alternative ATG start sites. Each of these variants potentially has catalytic activity since, in each case, the lipase active site is not modified by the splicing or truncation. In this embodiment, expression of the full-length form (SEQ ID NO: 13) encoding the 88kDa polypeptide (SEQ ID NO: 1) results in expression of truncated isoforms with catalytic activity in both a baculoviral expression system and in transgenic mice.

[000103] The iPLA$_2\gamma$ polynucleotides of the present invention include those nucleic acid molecules, both DNA and RNA, which encode the iPLA$_2\gamma$ polypeptides. Such iPLA$_2\gamma$ polypeptides include, in particular, the coding regions of splice variants as set forth in SEQ ID NOS 3 and 4 (splice variants gamma 1 and gamma 2) and SEQ ID NO:5 (splice variant gamma 3) (see Figures 2-4) as well as their complements, SEQ ID NOS 7, 8 and 9, respectively. Also included are cDNA sequences which show at least 20% identity with the cDNA sequences of SEQ ID NOS 3, 4, 5, 6, 7, 8 or 9 as defined above and sequences which specifically hybridize to SEQ ID NOS 3, 4, 5, 6, 7, 8 or 9.

[000104] The phrase "by specifically hybridizing to said sequences", is meant that a given sequence hybridizes under high stringency conditions. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. Such factors are outlined in, for example, Sambrook et al. (Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). High stringency conditions serve to prevent false positives, that is the hybridization and apparent detection of iPLA$_2\gamma$ polynucleotide sequences when in fact an intact and functioning iPLA$_2\gamma$ polynucleotide is not present.

[000105] Probes and primers which are capable of hybridizing to an iPLA$_2\gamma$ sequence are also included within the present invention. Probes can be oligonucleotides of from about 25 to about 100 nucleotides in length which specifically hybridize to a target sequence. Primers within the scope of the present invention are typically short strands of DNA or RNA having a length of at least from about 6 to about 8 nucleotides, more preferably about 12 nucleotides, still more preferably about 15 nucleotides, and most preferably about 20 to about 30 nucleotides or greater in length. Such nucleic acid sequences hybridize to the target region. The primers herein are selected to be "substantially" complementary to

different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

[000106] The present invention includes fragments and truncated polynucleotides which can be used in modulating the translational regulation of $iPLA_2\gamma$, and in the identification of naturally occurring repressor substances within the cell. Thus, by cloning of portions of the wild type $iPLA_2\gamma$ polynucleotide into a vector one can identify those portions of the sequence involved in transcriptional or translational regulation within the cell. These polynucleotide portions of the $iPLA_2\gamma$ sequence can bind to naturally occurring repressor substances in a cell and serve as binding ligands to the repressor substance. Such binding can block the decrease in $iPLA_2\gamma$ polynucleotide expression action that would otherwise occur if the repressor substance were free to bind to the $iPLA_2\gamma$ polynucleotide. In addition, the binding to a repressor substance by the polynucleotides can serve as a basis for identifying and isolating new compounds which prevent the binding of the repressor substance to $iPLA_2\gamma$ polynucleotides. Such binding to the repressor substance produces a de-repressor action to allow an increased expression of $iPLA_2\gamma$ polynucleotides.

[000107] The present invention also includes treatment of certain diseases using $iPLA_2\gamma$, protein products thereof, or a translational repressor molecule thereof. Based on its genomic characterization and biological activity, $iPLA_2\gamma$ is a likely candidate for involvement in a number of disease processes. Without being bound to a particular theory, the inventors herein have discovered that $iPLA_2\gamma$ is a likely candidate for involvement in Alzheimer's disease because its sn-2-lipase activity regulates the release of lipid second messengers. Such second messengers include arachidonic acid and its eicosanoid metabolites which are prominent lipid second messengers for inflammatory processes. (See e.g. Needleman et al., supra). It has been previously established that the neural damage underlying Alzheimer's disease ultimately results from a final common pathway involving precipitation of amyloid protein in a plaque formation that initiates lipid inflammation and produces dystrophic

changes in the affected neurons. The amyloid plaques of Alzheimer's disease are a nidus for localized inflammation, and the neural dystrophic process in Alzheimer's disease is characterized by the presence of several biochemical markers of inflammation, and inflammation may be the proximal cause of synaptic loss during neural dystrophy. (See e.g. Farooqui et al., supra). Thus, the inventors herein have, in identifying and characterizing $iPLA_2\gamma$, also succeeded in identifying $iPLA_2\gamma$ as a new candidate for mediating inflammatory processes involved in Alzheimer's disease. Treatment for Alzheimer's disease would thus involve any treatment that decreases the activity of $iPLA_2\gamma$, especially in brain regions affected by Alzheimer's disease.

[000108] Similarly, $iPLA_2\gamma$ may be regarded as a candidate for mediating other inflammatory processes such as asthma.

[000109] The $iPLA_2\gamma$ polypeptides of the present invention can also be used in the treatment of atherosclerotic heart disease, which represents a major cause of morbidity and mortality in the United States and other industrialized nations. In patients with myocardial infarction, the infarct size and type (i.e. transmural or subendocardial) profoundly affect realignment and remodeling of affected myocardial zones, and hence affect whether the infarct results in heart failure, or merely electrophysiologic dysfunction. Recent experimental results have shown that agents that enhance the efficiency of ATP production from the available O2 supply have a salutary effect on the size and type of myocardial infarction. These findings implicate the phenomenon known as oxygen wasting, and pose as therapeutic candidates any agents that may have a beneficial effect on the efficiency of O2 utilization by reducing oxygen wasting. In normal myocardium, most fatty acid oxidation occurs in the mitochondrial compartment, while a smaller but not insignificant portion of fatty acid oxidation, about 3-4%, occurs in peroxisomes. In the peroxisomal compartment, fatty acid oxidation occurs without efficient production of reducing equivalents but heat and resulting in oxygen wasting. During myocardial ischemia, mitochondrial oxidation of fatty acids is impaired to about 10% of normal levels. Thus, the oxygen wasted in the peroxisomal compartment gains greater significance because oxygen availability becomes rate-limiting. Therefore, reduction in oxygen wasting in the peroxisomal compartment during myocardial ischemia is likely to profoundly benefit myocyte preservation with attendant desirable effects on the clinical sequelae of ischemia or infarct. Thus, the inventors herein have, in identifying and characterizing $iPLA_2\gamma$, have also succeeded in identifying $iPLA_2\gamma$ as a new candidate

target for limiting oxygen wasting. Treatment for myocardial ischemia or myocardial infarction would thus also involve any treatment that decreases the activity of $iPLA_2\gamma$, especially in ischemic heart tissue.

[000110] One approach for decreasing the activity of $iPLA_2\gamma$ is by the identification and administration to a living cell, a translational repressor substance which decreases translation of $iPLA_2\gamma$ or administration of the nucleic acid encoding the translational repressor substance. Such repressor molecules can be identified by virtue of their binding to translational repressor binding sites identified herein.

[000111] Alternatively, $iPLA_2\gamma$ activity can be decreased by treatment with $iPLA_2\gamma$ antisense oligonucleotides. Such antisense oligonucleotides interact through base pairing with a specific complementary nucleic acid sequence involved in the expression of $iPLA_2\gamma$ such that the expression of $iPLA_2$ is reduced. The $iPLA_2\gamma$ antisense oligonucleotides preferably comprise a sequence containing from about 8 to about 100 nucleotides and more preferably the $iPLA_2\gamma$ antisense oligonucleotides comprise from about 15 to about 30 nucleotides. The $iPLA_2\gamma$ antisense oligonucleotides can also contain a variety of modifications that confer resistance to nucleolytic degradation such as, for example, modified internucleotide lineage's (Uhlmann and Peyman, Chemical Reviews 90:543-548 1990; Schneider and Banner, Tetrahedron Lett. 31:335, 1990), modified nucleic acid bases and/or sugars and the like.

[000112] Agents that regulate or control peroxisomal ß-oxidation are also potentially useful in controlling the related diseases of obesity, hyperlipidemia and diabetes, which represent major health problems in the United States and other industrialized nations. Obesity and hyperlipidemia are primary features of the metabolic syndrome, which identifies those at high risk for developing insulin-resistant (Type II) diabetes. Obesity and high serum lipid levels also predispose individuals to developing atherosclerosis and hypertension. Accordingly, agents that decrease body weight, serum lipid levels or the insulin-resistance characterizing Type II diabetes, are avidly sought. One type of such agents operates by increasing caloric expenditure or fatty acid α and β-oxidation, and significant reductions in body weight can be achieved with only small increases (about 2-3%) in caloric expenditure. Since fatty acid β-oxidation in peroxisomes wastes oxygen, producing only heat, and no energy in the form of NADH or ATP, an agent that thus increases caloric expenditure by increasing peroxisomal fatty acid β-oxidation, directly or indirectly, has potentially major

beneficial effects in the treatment of obesity, hyperlipidemia, diabetes, atherosclerosis and hypertension. Thus, the inventors herein have, in identifying and characterizing $iPLA_2\gamma$, also succeeded in identifying $iPLA_2\gamma$ as a new candidate target for increasing caloric expenditure by increasing peroxisomal fatty acid γ and β-oxidation. Accordingly, treatment for the related diseases of diabetes, obesity, and hyperlipidemia would thus also involve any treatment that modulates the activity of $iPLA_2\gamma$, especially in Type II diabetic or obese individuals.

[000113] The present invention, therefore, also includes therapeutic or pharmaceutical compositions comprising $iPLA_2\gamma$ polypeptides and polynucleotides as well as a blocker of $iPLA_2\gamma$ translational repressor molecule, in an effective amount for treating patients with inappropriately low levels of $iPLA_2\gamma$. Such diseases include but are not limited to Type II diabetes, obesity and hyperlipidemia. The treatment can comprise administering a therapeutically effective amount of $iPLA_2\gamma$ polynucleotide or $iPLA_2\gamma$ protein or an effective amount of blocker of $iPLA_2\gamma$ translational repression.

[000114] The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including for example intravenous, subcutaneous, intramuscular, transdennal, intrathecal or intracerebral. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation.

[000115] $iPLA_2\gamma$ can also be linked or conjugated with agents that provide desirable efficiencies pharmaceutical or pharmacodynamic properties. For example, $iPLA_2\gamma$ can be coupled to any substance known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor, and administered by intravenous injection (See, for example, Friden et al., Science 259:373-377, 1993). Furthermore, $iPLA_2\gamma$ can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See, for example Davis et al. Enzyme Eng 4:169-73, 1978; Burnham, Am J Hosp Pharm 51:210-218, 1994).

[000116] In an aspect, the compositions are employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. In an aspect, one preparation utilizes a vehicle of physiological saline

solution or, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. Calcium-independent phospholipase $A_2\gamma$ can also be incorporated into a solid or semi-solid biologically compatible matrix that can be implanted into tissues requiring treatment.

[000117] The carrier can also contain other pharmaceutically-acceptable excipients and additives for modifying or maintaining pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion into the cerebrospinal fluid by continuous or periodic infusion. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

[000118] It is also contemplated that certain formulations containing $iPLA_2\gamma$ are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl-and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and promote absorption such as, for example, surface active agents.

[000119] The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in assay preparations of target cells. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

[000120] In a number of circumstances it would be desirable to determine the levels of containing iPLA$_2\gamma$ in a patient sample. The term "detection" as used herein in the context of detecting the presence of iPLA$_2\gamma$ in a patient is intended to include determining the amount of iPLA$_2\gamma$ or the ability to express an amount of iPLA$_2\gamma$ in a patient, the distinguishing of iPLA$_2\gamma$ from other phospholipases A$_2$, the estimation of prognosis in terms of probable outcome of a degenerative disease and prospect for recovery, the monitoring of iPLA$_2\gamma$ levels over a period of time as a measure of status of the condition, and the monitoring of iPLA$_2\gamma$ levels for determining a preferred therapeutic regimen for the patient.

[000121] To detect the presence of iPLA$_2\gamma$ in a patient, a sample is obtained from the patient. The sample can be a tissue biopsy sample or a sample of blood, plasma, serum, CSF (cerebral spinal fluid) or the like. Samples for detecting iPLA$_2\gamma$ can be taken from any of these tissues. To detect the presence of iPLA$_2\gamma$, an enzymatic analysis or Western blot analysis can be performed.

[000122] The term "probe" as used herein refers to a structure comprised of a polynucleotide which forms a hybrid structure with a target sequence, due to complementarity of probe sequence with a sequence in the target region. Oligomers suitable for use as probes may contain a minimum of about 8-12 contiguous nucleotides which are complementary to the targeted sequence and preferably a minimum of about 20, more preferably a minimum of about 25, still more preferably a minimum of about 30, yet still more preferably a minimum of about 40, and most preferably a minimum of about 50.

Calcium-independent $A_2\gamma$ gene probes of the present invention can be DNA or RNA oligonucleotides and can be made by any method known in the art such as, for example, excision, transcription or chemical synthesis. Probes may be labeled with any detectable label known in the art such as, for example, radioactive or fluorescent labels or enzymatic marker. Labeling of the probe can be accomplished by any method known in the art such as by PCR, random priming, end labeling, nick translation or the like. One skilled in the art will also recognize that other methods not employing a labeled probe can be used to determine the hybridization. Examples of methods that can be used for detecting hybridization include Southern blotting, fluorescence, in situ hybridization, and single-strand conformation polymorphism with PCR amplification.

[000123] Hybridization is typically carried out at 25-45°C, more preferably at 32-40°C, and more preferably at 37-38°C. The time required for hybridization is from about 0.25 to about 96 hours, more preferably from about 1 to about 72 hours, and most preferably from about 4 to about 24 hours.

[000124] The PCR method for labeling probes or amplifying target sequences uses primers that flank or lie within the target, here $iPLA_2\gamma$ gene. The PCR method is well known in the art. Briefly, PCR is performed using two oligonucleotide primers which are capable of hybridizing to the nucleic acid sequences flanking a target sequence that lies within a $iPLA_2\gamma$ gene and amplifying the target sequence. The terms "oligonucleotide primer" as used herein refers to a short strand of DNA or RNA ranging in length from about 8 to about 50 bases. The upstream and downstream primers are typically from about 20 to about 30 base pairs in length and hybridize to the flanking regions for replication of the nucleotide sequence. The polymerization is catalyzed by a DNA-polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs to produce double-stranded DNA molecules. The double strands are then separated by any denaturing method including physical, chemical or enzymatic. Commonly, the method of physical denaturation is used involving heating the nucleic acid, typically to temperatures from about 80°C to 105°C for times ranging from about 1 to about 10 minutes. The process is repeated for the desired number of cycles.

[000125] The primers are selected to be substantially complementary to the strand of DNA being amplified. Therefore, the primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with the strand

being amplified. Preferably, primers are at least 10 nucleotides long, more preferably at least 15 nucleotides long, still more preferably at least 20 nucleotides long, still more preferably at least 25 nucleotides long, still more preferably at least 30 nucleotides long, still more preferably at least 40 nucleotides long, and most preferably at least 50 nucleotides long. After PCR amplification, the DNA sequence comprising $iPLA_2\gamma$ or a fragment thereof is then directly sequenced and analyzed.

[000126] Exemplary embodiments of the invention are described in the following examples. Other alternative embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

[000127] The following methods were used in the examples below. Specific materials used in the work reported herein were as described herein, but those skilled in the art will recognize that many suitable alternative materials are readily available from commercial suppliers. [α-^{32}P]dCTP (6000 Ci/mmol) and ECL detection reagents were purchased from Amersham Pharmacia Biotech. A human heart cDNA library was purchased from Stratagene, Inc. Human heart Marathon cDNA, QuickClone human skeletal cDNA, and human MTN multiple tissue Northern blots were purchased from CLONTECH. For PCR, a Perkin-Elmer Thermocycler was used, and all PCR reagents were purchased from PE Biosystems. The pGEM-T vector and TnT Quick Coupled Transcription/Translation System were purchased from Promega. Vector pcDNA1 .1 was purchased from Invitrogen. Culture media, Cellfectin and LipofectAMINE reagents for transfection of baculovirus vectors, and competent DH110Bac Escherichia coli cells were purchased from Life Technologies, Inc., and used according to the manufacturer's protocol. QIAfilter plasmid kits and QIAquick gel extraction kits were obtained from Qiagen, Inc. Keyhole limpet hemocyanin was obtained from Pierce. L-α-1-dipalmitoyl-2-[1-^{14}C] phosphatidylcholine, L-α-1-palmitoyl-2-[1-^{14}C] linoleoyl phosphatidylcholine, and L-α-1-palmitoyl-2-[1-^{14}C] linoleoyl phosphatidylcholine, and L-α-1-palmitoyl-2-[1- αC] arachidonyl phosphatidyleholine were purchased from NEN Life Science Products. 1-O-(Z)-hexadec-1'-enyl-2-[9,10-3H]octadec-9'-enoyl-sn-glycero-3-phosphocholine was synthesized and purified as described in Han et al., Anal. Biochem. 200:119-24, 1992, which is herein incorporated by reference. The luciferase assay kit used

was that of Promega. BEL was obtained from Sigma. Any other reagents were also obtained primarily from Sigma. Searches of EMBL and NCBI databases were performed using the Basic Local Alignment Search Tool (BLAST) (NCBI). Alignments of all sequences were performed using the MultiAlign computer program, as described in Corpet, F., Nucleic Acids Res. 16:10881-90, 1988, which is herein incorporated by reference.

[000128] Expression of iPLA$_2\gamma$ in an intact eukaryotic system was evaluated using cultured Spodoptera frugiperda (Sf9) cells. Sf9 cells have been widely used to express recombinant proteins as a host cell in the baculovirus expression system. (See e.g. Liu et al., J. Immunol. 156:3292-300, 1996). It is believed that the translational elements of Sf9 cells exhibit a high degree of homology with mammalian translational elements. (See e.g. Sakahira et al., .J. Bio. Chem. 274:15740-4, 1999; Kimball et al., J. Biol. Chem. 273:12841-5, 1998; Gu et al., Biochim. Biophys. Acta 1399:1-9, 1998). Sf9 cells were grown and infected with wild-type or recombinant baculovirus containing human phospholipase A2γ cDNA as described in detail in Wolf, M.J. and Gross, R.W., J. Bio. Chem. 27 1:30879-85, 1996, which is herein incorporated by reference. Culture of Sf9 cells involved, in brief, placing Sf9 cells in growth medium in 100-ml spinner flasks equipped with a magnetic spinner. The rotation speed of the magnetic spinner was set at about 80 rpm, which is believed to be especially suitable for optimizing growth of Sf9 cells. The growth medium included Grace's medium supplemented with Yeastolate, Lactalbumin, 1% Antimycotic (all from Sigma), 10% fetal bovine serum and 1% Plutonic F-68 surfactant. Sf9 cells at a concentration of 1x106 cells/ml were prepared in 50 ml of the growth medium and incubated at 27°C for one (1) hour prior to infection with either wild-type baculovirus or recombinant baculovirus containing human phospholipase A$_2\gamma$ cDNA. After 48 hours, cells were repelleted by centrifugation, resuspended in ice-cold phosphate-buffered saline, and repelleted. All subsequent operation were performed at 4°C. The supernatant was decanted, and the cell pellet was re-suspended in 5 ml homogenization buffer (25 mM imiazole, pH 8.0, 1 mM EGTA, 1mM dithiothreitol, 0.34 M sucrose, 20 MM transepoxysuccinyl-L-leucylamido-(4-guanidino) butane, and 2 /ml leupeptin. Cells were lysed at 0°C by sonication (twenty (20) one-second bursts utilizing a Vibra-cell sonicator at a 30% output) and centrifuged at 100,000 x g for one (1) hour. The supernatant containing predominantly cytosol was saved, and the membrane pellet was washed with homogenization buffer and resuspended using a Teflon homogenizer in 6 ml of homogenization buffer. After a brief sonication (ten (10) one-second bursts), the mixture was subjected to recentrifugation at 100,000 x g for one (1) hour. After removal of the

supernatant, the membrane pellet was resuspended in 1 ml homogenization buffer using a Teflon homogenizer and subsequently sonicated at 0°C for five (5) one-second bursts.

[000129] For PCR, primer design and selection of appropriate restriction sites for constructing vectors proceeded according to the following. The free Internet software program Amplify 1.2 was used to design nucleotides containing appropriate terminal restriction sites. In general, oligonucleotides are at least 20 nucleotides long, with approximately 50% GC/AT ratio, and do not have a T at the 3' end. The Amplify program predicts the efficiency of nucleotide priming, the presence of primer dimer pairing, if any, and the predicted sizes and approximate amounts of products obtained. The $iPLA_2\gamma$ used for designing primers has been deposited in the GenBank® database (accession number AF2636 13).

[000130] For PCR amplification of $iPLA_2\gamma$, the reagents (10x PCR buffer, Mg buffer, dNTPs, and Amplitaq) were obtained from PE Biosystems. Plugged pipette tips were used in all PCR procedures to minimize cross-contamination. PCR amplification occurred in the Perkin Elmer model 460 thermocycler. PCR reactions (50μl) were set up as follows:

10x PCR buffer	5 μl
Mg	3 μl
10 mM dNTO mix	1 μl each
Sense primer	0.5 μl of 0.08 mM stock
Antisense primer	0.5 μl of 0.08 mM stock
DNA template 1	1 μl of miniprep (from a 50 μl miniprep stock)
Sterile millipore water	35.75μl

[000131] The thermocycler was programmed to heat the above reaction mixture for five minutes at 94°C followed by two minutes at 80°C during which 0.25μl Amplitaq and one drop of oil were added prior to initiation of the cycling program. For PCR analysis, a 30-cycle program was employed, with steps at 53°C for 30 seconds, 72°C for two minutes, and 94°C for 30 seconds per cycle. IPLA2γ was amplified utilizing oligonucleotides that flanked the predicted 5'- and 3'-coding region, M444 (5'-

TTTTGTCGACATGTCTATTAATCTGACTGTAGATA-3')(SEQ ID NO:11) and M449 (5'-GCATACTCGAGTCACAATTTTGAAAAGAATGGAAGTCC-3') (SEQ ID NO:23), respectively to engineer appropriate restriction sites onto iPLA2γ for subsequent cloning into SalI/SphI restriction sites of a pFASTBAC vector (Life Technologies, Inc.). PCR screening was performed using human skeletal muscle cDNA (0.5 ng), human heart Marathon cDNA (0.5 ng) and a human heart cDNA library (approximately lxl09 plaque-forming units) as templates. To directly compare differences between the sequences reported herein and previously reported sequences, PCR amplification of sequence previously reported in a BAC genomic clone (Research Genetics) was used as a template, and PCR was performed with primers M452 (5'-GTACATACGGTGGACAAGCCTA-3')(SEQ ID NO:24) and M446 (5'-CATTCCTCTCCCTTTCACTGGATCCACATAGCC-3')(SEQ ID NO:25).

[000132] PCR products were resolved by 1% agarose gel electrophoresis. Candidate bands visualized with ethidium bromide staining were extracted from the agarose gel using the QIAquick Gel extraction kit from Qiagen. The extracted DNA and cloning vector (pFASTBAC) were restriction digested with appropriate restriction enzymes (SaII/SphI for the above primers), resolved on a 1 % agarose gel, extracted with the QIAquick Gel extraction kit, and then ligated. In a typical ligation 2 to 10 molar excess restriction digested PCR product was ligated overnight at 16°C to restriction digested vector in a 20μl reaction using T4 DNA ligase (Promega). For transformation, Stratagene XL2-Blue MRF ultracompetent cells (#200151) were used according to the manufacturer's protocol. Following bacterial transformation and growth of transformants, plasmids were purified using a QIAfilter plasmid kit from Qiagen, and subjected to automated sequence analysis using either an ABI 373S or 377XL automated DNA sequencer (PE Biosystems).

[000133] Bacmids were prepared from the pFASTBAC plasmid constructs using the Bac-to-Bac Baculovirus Expression System protocol from Life Technologies, Inc. In brief, pFASTBAC plasmid constructs were used to transform DH1OBac competent cells resulting in a Tn7 site-specific transposition to produce a composite bacmid containing the cloned sequence of interest. This bacmid was used in Cellfectin-mediated transfection of Sf9 cells in a 35 mm plates for 72 hours resulting in production of infectious recombinant baculovirus in the Sf9 cells. Recombinant baculovirus (1 ml) obtained was used to infect 50 ml of Sf9 cells in a 100 ml spinner for an additional 72 hours to provide a stock of amplified virus.

[000134] To prepare truncated iPLA2γ constructs in pFASTBAC, PCR primers containing appropriate restriction sites for cloning were used. For example, the original full-length construct was used as a template in PCR with a sense primer annealing at nt 650-676 (SEQ ID NO:20) paired with reverse primer M458 (SEQ ID NO: 12) to create a truncated iPLA2γ (63kDapF) with a methionine start site corresponding to amino acid 221 in the full-length protein, expression of this construct in the baculovirus system resulted in a 63 kDa recombinant protein. A second construct was created with a start site corresponding to methionine 122 to produce a 74 kDa recombinant protein (SEQ ID NO. 18) using PCR primers corresponding to SEQ ID NOS 17 and 12. Similarly, PCR primers M534 (SEQ ID NO: 14) and M458 (SEQ ID NO: 12) were used to prepare a PCR product containing the 77kD coding sequence (SEQ ID NO: 16) for cloning into pFASTBac vector and subsequent expression of the 77kDa polypeptide (SEQ ID NO: 15) in the baculoviral expression system. Segments of iPLA2γ 5' sequence ranging between 15-663 nt were inserted 5' of the insert sequence in 63kDapF to identify potential sequences involved in translational regulation. These sequences were ligated as in frame 5' coding sequence or as 5' noncoding sequence to the 63kDapF construct. For recombinant protein produced in the Sf9 system as described herein, enzymatic activity was determined using the enzyme assay as described below in Example 6, and mass of each of these constructs was determined using Western analysis as described herein. Additionally, full-length truncated $iPLA_2\gamma$ and truncated $iPLA_2\gamma$ constructs were inserted 5' of full-length luciferase coding sequence which was cloned into pFASTBAC via NotI/XbaI restriction sites. Luciferase activity of recombinant protein produced in the Sf9 system was then measured using the Luciferase Assay System of Promega, as described in detail in Example 8 below. (Wood, K.V., (1991) In: Bioluminescence and Chemiluminescence: Current Status, Stanley, P. and Kricka, L. eds., John Wiley and Sons, Chichester, NY, 543; and Luciferase Assay System Technical Bulletintus, #TB 101, Promega Corporation, 1996, which are herein incorporated by reference).

[000135] Furthermore, each of the truncated forms can be cloned into vectors (such as alpha MHC vector) for generation of transgenic mice expressing each truncated form as a recombinant protein. Constructs can also be modified by addition of appropriate tags (such as a 6xHis (SEQ ID NO: 54)) for generation of tagged recombinant protein facilitating their purification and characterization. The procedure for generation of transgenic mice is described in Example 11. Expression of full-length and truncated forms in transgenic mice will facilitate studies of the functions of $iPLA_2\gamma$ in whole animals (as opposed to in vitro or in

tissue culture cells) and will help elucidate the role of $iPLA_2\gamma$ in normal and abnormal physiology (e.g. lipid homeostasis, myocardial disorders, and diabetes).

[000136] The unique ability of antibodies to recognize and specifically bind to target proteins provides an approach for treating an over expression of the protein. Thus, another aspect of the present invention provides for a method for preventing or treating diseases involving over expression of the $iPLA_2\gamma$ protein by treatment of a patient with specific antibodies to the $iPLA_2\gamma$ protein. Specific antibodies, either polyclonal or monoclonal, to $iPLA_2\gamma$ protein can be produced by any suitable method known in the art. For example, the $iPLA_2\gamma$ protein, or an immunologically active fragment thereof, or an anti-idiotypic antibody, or fragment thereof can be administered to an animal to elicit the production of antibodies capable of recognizing and binding to the $iPLA_2\gamma$ protein. Such antibodies can be from any class of antibodies including, but not limited to IgG, IgA, IgM, IgD, and IgE or in the case of avian species, IgY and from any subclass of antibodies. Polyclonal antibodies can be prepared by immunizing rabbits or other animals by injecting antigen followed by subsequent boosts at appropriate intervals. The animals are bled and sera assayed against purified $iPLA_2\gamma$ protein usually by ELISA or by bioassay based upon the ability to block the activity of $iPLA_2\gamma$ protein in cells. When using avian species, e.g. chicken, turkey and the like, the antibody can be isolated from the yolk of the egg. Monoclonal antibodies can be prepared after the method of Milstein and Kohler by fusing splenocytes from immunized mice with continuously replicating tumor cells such as myeloma or lymphoma cells. (Milstein and Kohler, Nature 256:495-497, 1975; Gulfre and Milstein, METHODS IN ENZYMOLOGY: IMMUNOCHEMICAL TECHNIQUES 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). The hybridoma cells so formed are then cloned by limiting dilution methods and supernates assayed for antibody production by ELISA, RIA or bioassay.

[000137] More specifically, to generate and purify the anti-$iPLA_2\gamma$ peptide polyclonal antibodies used in the work described herein, New Zealand white rabbits were immunized with the $iPLA_2\gamma$ synthetic peptide CENIPLDESRNEKLDQ (SEQ ID NO:26). This peptide (G3, 4 mg), dissolved in minimal volume of dimethylsulfoxide, was conjugated to maleimide-activated keyhole limpet hemocyanin (KILH, 2 mg) in 200 1d of 83 mM sodium phosphate, 0.9 M NaCl , and 0.1 mM EDTA at 22°C for two hours. After extensive dialysis against 83 mM sodium phosphate containing 0.9 mM NaCl, a 1:1 emulsion was

made with the peptide-KLH conjugate and Freund's complete adjuvant. This emulsion was then injected subcutaneously into two anaesthetized New Zealand white rabbits. Two booster injections of a 1:1 emulsion of the peptide-KLH conjugate and Freund's incomplete adjuvant were given two and four weeks after the initial immunization. Serum was then collected for purification of the anti iPLA2γ antibodies.

[000138] A peptide affinity column was generated by coupling the iPLA$_2\gamma$ synthetic peptide to a thiopropyl-Sepharose (Amersham-Pharmacia) resin in the presence of 100 mM sodium citrate, pH 4.5. The resin-peptide suspension was mixed by inversion overnight at 22 °C. Coupling efficiency was monitored by the displacement of the 2-pyridyl sulfide groups as 2-thippyridone through spectrophotometric monitoring at 343 mn. Before chromatography, the resin-peptide suspension was equilibrated with 10 mM Tris-HCl, pH 7.5 in an Econo-pac column (BioRad) by gravity flow. Serum containing the anti the iPLA2γ antibodies was diluted 1:10 (v/v) with 10 mM Tris-HCl, pH 7.5 and then applied to the peptide affinity column by gravity flow. The diluted serum was then re-applied twice to the column to assure binding of the antibody to the column. After washing the column extensively with 10 mM Tris-Hcl, pH 7.5 containing 500 mM NaCl, bound anti-iPLA2γ antibody was eluted from the column with 100 mM glycine, pH 2.5 and the immediately neutralized with 100 mM Tris-Hcl, pH 9.0. The antibody was then concentrated using Centricon-10 (Americon) centrifugal filter devices and stored at 40°C until use.

EXAMPLE 1

[000139] This example illustrates the approach used in identifying the full-length cDNA encoding phospholipase A$_2\gamma$.

[000140] Inspection of a sequence encoding a putative 40kDa phospholipase reported by the Human Genome Sequencing Project (BAC clone RG054D04; GenBank® accession no. AC005058) demonstrated that the sequence did not begin with an initiator methionine codon. Thus, a TBLASTN data base search was undertaken to find expressed sequence tags (EST's) that might align with the 5'-end of the putative phospholipase sequence.

[000141] Six expressed sequence tags (EST's) identified in the TBLASTN search were mapped on the basis of their overlapping the 40kDa cDNA sequence or on the basis of their binding in close spatial proximity to the putative phospholipase sequence.

Figure 8 is a schematic illustration showing this mapping. Amino acid numbering starting from the most 5' potential translation site is indicated by the scale at top, with a schematic representation of the previously identified putative 40-kDa phospholipase shown shaded below. The EST sequences that were used to identify a potential N-terminal initiator methionine and to map the full-length sequence of a novel phospholipase, calcium-independent phospholipase $A_2\gamma$ (iPLA$_2\gamma$), are indicated by solid bars. The accession number for each EST sequence is indicated above each solid bar. The arrows at bottom indicate the position and orientation of PCR primers M444 (5'-end) and M449 (3'-end) used to amplify a full-length 2.4-kb coding sequence for iPLA2γ from human heart cDNA.

[000142] Specifically, EST clones vz36b01.ri Soares 2NbMT Mus musculus cDNA IMAGE:1328521 (accession no. AA915661) and Rattus norvegicus cDNA UI-R-C0-hp-c-06-0-UI (accession no. AA998901) were found to overlap with the BAC clone sequence, thereby adding an additional 360 nt sequence to the previously known 5' end of the putative phospholipase sequence. Four other EST clones found in the GenBank® database (Stratagene Homo sapiens colon cDNA clone IMAGE:5 88479, accession no. AA143503; Stratagene H. sapiens cDNA clone IMAGE:647744, accession no. AA205528; normalized rat ovary, Bento soares Rattus sp. cDNA clone ROVAA46, accession no. AA801084; and NCI_CGAP_GCB1 H. sapiens cDNA clone IMAGE:825005, accession no. AA504219) were found to be in close spatial proximity to the putative phospholipase sequence from the BAC clone. However, when aligned with the BAC clone sequence, the 3'-end of the EST AA504219 sequence and the 5'-end of EST AA998901 are separated by a 150-nt gap, as shown in Figure 8. Moreover, when the EST AA998901 sequence was back-translated through the gap and into EST AA504219, a continuous reading frame resulted. Thus, by overlapping known EST sequences and back-translating through the 150-nt gap, the inventors have succeeded in extending the putative phospholipase sequence approximately 1.2 kb upstream from the predicted GenBank® protein product.

[000143] The furthest upstream ATG codon that remained in frame with the newly identified full-length coding sequence was located 1210 nt upstream from the previously reported BAC clone sequence. Translation of the reported gene sequence further 5' from nt 122761 in BAC clone RGO54DO4 results in stop codons in all three reading frames.

[000144] Based upon the predicted sequence obtained from this assembly of sequences in the database, primers were constructed to obtain and sequence the putative full-length phospholipase. For PCR, primer design and selection of appropriate restriction sites for constructing vectors proceeded according to the following. The free software program Amplify 1.2 was used to design nucleotides containing appropriate terminal restriction sites. In general, oligonucleotides are at least 20 nucleotides long, with approximately 50% GC/AT ratio, and do not have a T at the 3' end. The Amplify program predicts the efficiency of nucleotide priming, the presence of primer dimer pairing, if any, and the predicted sizes and approximate amounts of products obtained. The $iPLA_2$ used for designing primers has been deposited in the GenBank® database (accession number AF263613).

[000145] PCR analysis used primers corresponding to the most 5' candidate initiator methionine and the known 3' stop codon (nt 79,673) in the gene sequence.

[000146] For typical PCR analysis, a 30-cycle program was employed with steps at 53°C for 30 seconds, 72°C for two minutes, and 94°C for 30 seconds per cycle. $iPLA_2\gamma$ was amplified utilizing oligonucleotides that flanked the predicted 5'-and 3'-coding region, M444 (5 '-TTTGTCGACATGTCTATTAATCTGACTGTAGATA-3') SEQ ID NO:11and M449 (5'-GCATACTCGAGTCACAATTTTGAAAAGAATGGAAGTCC-3') (SEQ ID NO: 23), respectively to engineer appropriate restriction sites onto iPLA2γ for subsequent cloning onto SaII/SphI restoration sites of a pFASTBAC vector (Life Technologies, Inc.).

[000147] PCR screening was performed using human skeletal muscle eDNA (0.5 ng), human heart Marathon cDNA (0.5 ng) and a human heart cDNA library (approximately l x 109 plaque-forming units) as templates. To directly compare differences between the sequences reported herein and previously reported sequences, PCR amplification of sequence previously reported in BAC genomic clone RGO54 D04 (Research Genetics) was used as a template, and PCR was performed with primers M452 (5'-GTACATACGGTGGACAAGCCTA-3')(SEQ ID NO:24) and M446 (5'-CATTCCTCTCCCTTTCACTGGATCCACATAGCC-3')(SEQ ID NO :25).

[000148] PCR products were resolved by 1% agarose gel electrophoresis. Candidate bands were extracted from the agarose gel using the QIAquick Gel extraction kit from Qiagen followed by blunt end ligation into the pGEM-T-Vvector (Promega) by standard procedures. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold

Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, which is incorporated by reference). Following bacterial transformation and growth of transformants, plasmids were purified using a QIAfilter plasmid kit from CLONTECH, and subjected to automated sequence analysis using either an ABI 373S or 377XL automated DNA sequencer (PE Biosystems).

[000149] Figure 9 shows the results of PCR amplification of the novel human iPLA2 sequence, $iPLA_2\gamma$. Specifically, PCR was performed using human heart cDNA (0.5 ng; lane 1 in Figure 9), a cDNA library prepared from human heart (lane 2), human skeletal muscle (lane 3), and a blank control (lane 4), as templates as described in detail above. PCR primer M444 was positioned at the 5'-end, and primer M449 positioned at the 3'-end of the $iPLA_2\gamma$ coding sequence, in 30 cycles of amplification at 53°C for 30 seconds, 72°C for 2 minutes, and 94°C for 30 seconds. PCR products were analyzed on a 1% agarose gel and visualized using ethidium staining. Molecular size markers are shown at left in kb. PCR analysis gave rise to a single band, which was 2.4 kb in length, as indicated by the arrow.

EXAMPLE 2

[000150] This example illustrates the determination and characterization of the full length amino acid sequence of $iPLA_2\gamma$, based on the PCR product described in Example 1, Figure 9.

[000151] First, the PCR product was cloned and the sequence analyzed. More specifically, the PCR product was subeloned into pGEM-T Vector and sequenced in both directions. Based on amino acid residue I being the initiator methionine, the message encoded a 782-amino acid polypeptide with a calculated molecular weight of 88,476.9. Contained within the sequence were an ATP binding motif at amino acid residues 449-45, and a lipase consensus sequence at amino acid residues 481-485, as well as multiple cAMP phosphorylation sites, PKC phosphorylation sites, CK2 phosphorylation sites, and a microbody C-terminal targeting sequence as determined by a Prosite pattern search (Hoflnann et al., Nucleic Acids Res. 27: 215-19, 1999; Bucher, P. and Bairoch, A. ISMB-94 Proceedings: 2nd International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA, pp. 53-61, AAAI Press, Menlo Park, CA, 1994, which are incorporated herein by reference).

[000152] To further analyze the deduced iPLA$_2\gamma$ amino acid sequence, a Kyte-Doolittle hydrophobicity analysis was performed on the sequence. (Kyte, J. and Doolittle, R.F., J. Mol. Biol. 157:105-132, 1982, which is herein incorporated by reference). Figure 11 shows a hydropathy plot of the deduced amino acid sequence of human iPLA$_2\gamma$. A window size of 17 amino acids was used. Negative values on the y-axis represent increasing hydrophobicity. Hydrophobicity analysis thus revealed that the putative iPLA$_2\gamma$ had two major hydrophobic domains, a first at the extreme putative N-terminus and a second centered near the lipase consensus sequence, at Ser483.

[000153] To determine whether the iPLA$_2\gamma$ PCR product represented the true 5'-end of the coding sequence, and to locate additional message sequence 5' of nt 122,761, 5'-RACE was performed using a reverse primer (M460) near the 5'-end of the 2.4 kb phospholipase A$_2\gamma$PCR product, and a sense primer (APl) to the adapter sequence flanking the cDNA template.

[000154] More specifically, for 5'-RACE, a 45-cycle program with steps at 58°C for 30 seconds, 72°C for 2 minutes, and 94°C for 30 seconds per cycle was employed. Human heart Marathon-ready cDNA was used as a template (0.5 ng) and primer Apl (CLONTECH) was paired with M460 (5'-GAAAACCTCTTTGTAGACTGATGTGGCTTATCCTCCAG3')(SEQ ID NO:27) to amplify products. Products were analyzed by electrophoresis utilizing a 1% agarose gel and visualized by ethidium bromide staining. PCR products were excised from the gel, purified with a QlAquick gel extraction kit, and subcloned into pGEM-T vector (Promega) for sequencing and alignment with the iPLA$_2\gamma$ sequence.

[000155] The full-length human iPLA$_2\gamma$ message sequence as determined using 5'-RACE includes a putative transcription initiation site, 5'-untranslated region, coding sequence, and 3'-untranslated region that together form a 3.4kb mature message. Thus, this maneuver extended the 5' sequence from the putative initiator methionine residue an additional 225 base pairs upstream. Because the 225-base pair sequence contained stop codons in all three reading frames, it is believed that no additional coding regions are present in the 225-base pair sequence. The most 5' ATG site that is in frame with the calcium-independent phospholipase A$_2\gamma$ is located at nt 226. Additional in-frame ATG sites are located at nt 526, 589 and 886 (based on SEQ ID NO:6 numbering). Initiation of polypeptide synthesis at these potential methionine start sites would result in polypeptides of 77, 74 and

63 kDa, respectively. At the 3'-end of the gene, a signal site for 3' poly(A) processing (AATAAA) was identified 757 nt 3' of the TGA stop signal. The actual cleavage site for poly(A) addition usually occurs 10-30 nt 3' of the poly(A) signal, and at this location, a CA is believed to be the preferred sequence immediately 5' to the cleavage site. Additionally, GU-rich or U-rich elements are also typically found downstream of the poly(A) site. Since a CA dinucleotide occurs 35 nt 3' of the poly(A) signal sequence in $iPLA_2\gamma$, and highly U-rich sequences occur at nucleotides 3339-3343 and at 3373-3392, the inventors have deduced that the likely point of poly(A) addition occurs following nucleotide 3372. Accordingly, these results identify a putative transcription initiation site, 5'-untranslated region, coding sequence, and 3'-untranslated region that together form a 3.4 kb mature message for $iPLA_2\gamma$.

[000156] To determine if the 3.4 kb message was the full-length or nearly full-length message, or if additional, as yet unidentified regions of the gene were transcribed to potentially serve a promoter function, Northern blot analysis of human $iPLA_2\gamma$ mRNA was performed on multiple tissues.

[000157] For Northern blot analysis, full-length of $iPLA_2\gamma$ amplified by PCR was prepared for use as a probe by radiolabeling with [^{32}P]dCTP for one (1) hour at 37°C in the presence of Ready-to-Go labeling beads (Amersham Pharmacia) according to the instructions provided by the manufacturer. The radiolabeled probe was purified by gel filtration employing a 1-ml Sepharose G-25 spin column. For Northern analysis, and MTN blot (CLONTECH) containing 2μg of poly(A)+ RNA/lane from human brain, heart, liver, lung, pancreas and placental tissue was prehybridized at 68°C for 30 minutes in hybridization buffer, hybridized for one hour at 68°C with 6 radiolabeled $iPLA_2\gamma$ (2 x 106 cpm/ml), and washed in 2xSSC and 0.1% SDS twice for 30 minutes, followed by two washes with 0.1xSSC and 0.1% SDS for 40 minutes each at 50°C as per the manufacturer's instructions. Hybridized sequences were identified by autoradiography for 16 hours.

[000158] Figure 12 shows the results of a Northern blot analysis of human $iPLA_2\gamma$ mRNA. The Northern blot was human mRNA obtained from the multiple tissues, and the tissue distribution of $iPLA_2\gamma$ mRNA thus examined by hybridization of the ^{32}P-labeled full-length $iPLA_2\gamma$ with approximately 2μg of human poly(A)+ mRNA, followed by autoradiography, as described above. Lane 1 shows the results from heart, lane 2 from brain, lane 3 from placenta, lane 4 lung, lane 5 from liver, lane 6 from skeletal muscle, lane 7 from

kidney, and lane 8 from pancreas. The positions of RNA size markers are shown. Relative size in kb is indicated at left based on the mobility of the RNA standard ladder.

[000159] Our northern blotting of human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas mRNA demonstrated that each of these tissues possessed a 3.4 kb message that tightly bound to the full-length ^{32}P-labeled probe. The largest amount of message was present in heart, followed by the placenta and skeletal muscle, with smaller amounts present in brain, kidney, pancreas, lung and liver. Within the limits of resolution of the Northern blotting technique, only a single band was identified. Thus, the results indicate that the message identified by the inventors and shown in Figure 12 is either full-length or nearly full-length. Multiple additional 5'-RACE reactions from multiple libraries did not identify any further sequence.

EXAMPLE 3

[000160] This example illustrates analysis of the complete genomic organization of the iPLA2γ gene. To determine the genomic organization of the full-length iPLA$_2\gamma$ gene, an examination was undertaken of sequence upstream from the putative start site of the BAC genomic clone (GenBank® accession number ACOO5 058).

[000161] Examination of the iPLA$_2\gamma$ sequence upstream from the putative transcription start site did not reveal the presence of either TATA box, nor CAAT box consensus sequences. The full-length message of iPLA$_2\gamma$ was aligned with BAC genomic clone RGO54DO4 sequence to determine the location of intron/exon boundaries. Two intron/exon boundaries were then identified within the 5'-untranslated region of the iPLA$_2\gamma$ sequence utilizing conventional AG/GT splicing rules.

[000162] Figure 13 is a schematic illustration of the human iPLA$_2\gamma$ gene, deduced by determining the locations of intron/exon boundaries. Intron/exon boundaries of the iPLA$_2\gamma$ gene are shown in scale (in kilobases). The thirteen (13) exons of the gene are indicated as open boxes. Spaces between the exons represent the relative sizes of the twelve (12) introns contained with in the gene. Regions of the gene that correspond to the ATP binding, lipase and peroxisomal localization consensus sequences are indicated in exons 7, 8 and 13, respectively. The dashed box enclosing exons 5-11 corresponds to previously identified exons in the BAC genomic clone (GenBank® accession number ACOO5058). The boxes at the bottom indicate the nucleotide numbers corresponding to the original BAC

genomic clone report, with the sizes of each exon in nucleotides and in amino acids shown within.

[000163] More specifically, exon 1 of the iPLA$_2\gamma$ gene is 346 nt in length, (nucleotides 135322-135627 of BAC genomic clone RGO54DO4) and is followed by a 1.9 kb intron. Exon 2 of the iPLA2γ gene is 96 nt in length, (nucleotides 133,299-133,394 of BAC genomic clone RGO54DO4) and is followed by a 4.5-kb intron. Exon 3 is 46 nt in length, (nucleotides 128,746-128,791 of BAC genomic clone RGO54DO4) and is followed by a 5.9-kb intron. Exon 4 of the iPLA2γ gene is 112 nt in length, (nucleotides 125571-125460 of BAC genomic clone RGO54DO4) and is followed by a 3.2 kb intron. The first candidate ATG start site occurs 84 nt downstream from the start of exon 5 (nucleotides 121,692-122,844 of BAC genomic clone RGO54DO4), which is 1151 nt in length and followed by a 139-nt intron 3. Exon 6 is 139 nt in length, (nucleotides 121,411-121,522 of BAC genomic clone RGO54DO4) and is followed by an 11.5-kb intron. Exon 7 begins the coding sequence previously reported in the GenBank® BAC clone report, as indicated in Figure 13. Based on these findings, the iPLA2γ gene on chromosome 7 is ~56 kb in size and contains 13 exons, ranging in size from 46 to 1139 nucleotides. Further, the results indicate that both the ATP binding motif (amino acid residues 449-454), and the lipase consensus sequence (amino acid residues 481-485) of the iPLA2γ gene require splicing of adjacent exons to form complete functional sequences. This splicing requirement distinguishes iPLA$_2\gamma$ from the iPLA$_2\beta$, which contains its functional ATP and lipase sites within a single exon.

[000164] Still further, these results show that the promoter region of the iPLA$_2\gamma$ gene contains neither a TATA box nor a CCAAT sequence within 400 nucleotides flanking the putative transcription start site. However, Tfsearch analysis reveals other promoter elements (e.g. several Spl/GC boxes) that are located in the promoter region, as well as several potential cap signals. One of the potential cap signals is located immediately upstream of the predicted transcription initiation site (i.e. at residues -6 to -13). TATA-less promoters are generally thought to typically respond to a variety of stimuli, with a range of transcription regulatory responses including differential expression during embryogenesis, tissue-specific distribution, and regulation by either viral or pharmacologic stimuli. Many TATA-less genes are growth-regulated, so that low levels of gene expression are seen in non-growing cells but then expression up-regulated as cells proliferate. Thus, these results

provide a foundation for studies in which the identified promoter region is fused to a reporter gene and subsequently dissected to determine important transcriptional regulatory elements by deletional mutagenesis.

[000165] To investigate the possible existence of iPLA$_2\gamma$ splice variants including alternative locations of intron/exon boundaries, further analysis of the iPLA$_2\gamma$ sequence upstream from the putative transcription start site of the BAC genomic clone was undertaken. Three alternative splice variants were discovered. Figure 2 is a schematic illustration of the three alternative splice variants of the iPLA$_2\gamma$ gene, deduced in this further examination. A region of variation, including exons 2, 3 and 5 as identified above, is shown. Labeling indicates the size of corresponding protein in kDa, and length in nucleotides (nt). Open boxes are noncoding regions, shaded regions are putative coding regions, and stippled lines represent intron splicing. The three splice variants included a first, "Gamma 1", that includes exon 2, exon 3 and a portion of exon 5 in noncoding region, with the start site in exon 5. A second, "Gamma 2" variant, includes exon 2 and a portion of exon 5 in noncoding region, but excludes exon 3 altogether. A third, "Gamma 3" variant, includes a new potential ATG start site in exon 2, and thus a portion of exon 2, all of exon 3 and a truncated exon 5 in coding region.

EXAMPLE 4

[000166] To determine the molecular weight of the protein(s) translated by the iPLA$_2\gamma$ message, the 2.4 kb PCR product as described above was expressed in an in vitro reticulocyte lysate translation system, and the molecular weights of the protein products determined.

[000167] For in vitro translation, a full-length iPLA$_2\gamma$ construct in pcDNAl .1 (1μg) was used in a coupled transcription/translation rabbit reticulocyte translation lysate system (Promega) with RNA synthesis from the T7 promoter of pcDNA1.1 using T7 RNA polymerase and translation in the presence of 20 (μ)Ci of ~35 methionine for 90 minutes according to the manufacturer's instructions. More specifically, the full-length iPLA2γ PCR product was cloned into peDNAl .1 vector so that the T7 promoter region of pcDNAl .1 was upstream from the iPLA$_2\gamma$ sequence. For coupled in vitro transcription/translation, RNA was synthesized from 1 μg of iPLA$_2\gamma$ -pcDNAI.1 in the presence of T7 RNA polymerase. Translation of RNA in the rabbit reticulocyte lysate system was performed in the presence of [35S] methionine, and following translation, Std of labeled product was boiled for two

minutes in SDS loading buffer. Translated, radiolabeled protein products were then resolved by SDS gel electrophoresis and visualized by autoradiography.

[000168] Figure 14 shows the resolution by gel electrophoresis and visualization by autoradiography of two radiolabeled protein products of iPLA$_2\gamma$ in vitro translation. In vitro translated product corresponding to iPLA$_2\gamma$ is shown at left and indicated by "γ", and a negative control indicated by "ctl" is shown at right. More specifically, Figure 14 shows the resolution a first product corresponding to a molecular mass of approximately 77 kDa and a second corresponding to a molecular mass of approximately 63 kDa. Sizes of molecular weight standards in kDa are indicated at left.

[000169] The first radiolabeled protein product of about 77 kDa corresponds to translation initiation at methionine residue 101 of the full-length 88 kDa. The second radiolabeled protein product of about 63 kDa corresponds to translation initiation at methionine residue 221 of the full-length iPLA2γ.

EXAMPLE 5

[000170] To compare the results of in vitro translation as described in Example 4, with translation in an intact eukaryotic cell, the translation of 2.4 kb iPLA$_2\gamma$ in Sf9 cells was investigated.

[000171] This example thus illustrates the determination of the molecular weights of the two identified protein products of the iPLA$_2\gamma$ gene as expressed in an intact eukaryotic cell, that the observed products correspond to results obtained in the in vitro translation as described in Example 4, and that iPLA$_2\gamma$ protein products are present predominantly in the cell membrane.

[000172] Expression of iPLA$_2\gamma$ in an intact eukaryotic system was evaluated using cultured Spodoptera frugiperda (Sf9) cells. Sf9 cells have been widely used to express recombinant proteins as a host cell in the baculovirus expression system. (See e.g. Liu et al., J. Immunol. 156:3292-300, 1996). It is believed that the translational elements of Sf9 cells exhibit a high degree of homology with mammalian translational elements. (See e.g. Sakahira et al., J. Biol. Chem. 274:15740-4, 1999; Kimball et al., J. Biol. Chem. 273:12841-5, 1998; Gu et al., Biochim. Biophys. Acta 1399:1-9, 1998). Sf9 cells were grown and infected with wild-type or recombinant baculovirus containing human phospholipase A$_2\gamma$ cDNA as

described in detail in Wolf, M.J. and Gross, R.W., J. Biol. Chem. 271:30879-85, 1996, which is herein incorporated by reference. Culture of Sf9 cells involved, in brief, placing Sf9 cells in growth medium in l00-ml spinner flasks equipped with a magnetic spinner. The rotation speed of the magnetic spinner was set at about 80 rpm, which is believed to be especially suitable for optimizing growth of Sf9 cells. The growth medium included Grace's medium supplemented with Yeastolate, Lactalbumin, 1% Antimycotic (all from Sigma), 10% fetal bovine serum and 1% Plutonic F-68 surfactant. Sf9 cells at a concentration of 1x106 cells/ml were prepared in 50 ml of the growth medium and incubated at 27°C for one (1) hour prior to infection with either wild-type baculovirus or recombinant baculovirus containing human phospholipase $A_2\gamma$ cDNA. After 48 hours, cells were repelleted by centrifugation, resuspended in ice-cold phosphate-buffered saline, and repelleted. All subsequent operation were performed at 4°C. The supernatant was decanted, and the cell pellet was resuspended in 5 ml homogenization buffer (25 mM imiazole, pH 8.0, 1 mM EGTA, 1mM dithiothreitol, 0.34 M sucrose, 20 MM transepoxysuccinyl-L-leucylamido-(4-guanidino) butane, and 4g/ml leupeptin). Cells were lysed at 0°C by sonication (twenty (20) one-second bursts utilizing a Vibra-cell sonicator at a 30% output) and centrifuged at 100,000 x g for one (1) hour. The supernatant containing predominantly cytosol was saved, and the membrane pellet was washed with homogenization buffer and resuspended using a Teflon homogenizer in 6 ml of homogenization buffer. After a brief sonication (ten (10) one-second bursts), the mixture was subjected to recentrifugation at 100,000 x g for one (1) hour. After removal of the supernatant, the membrane pellet was resuspended in 1 ml homogenization buffer using a Teflon homogenizer and subsequently sonicated at 0°C for five (5) one-second bursts.

[000173] The 2.4-kb message encoding $iPLA_2\gamma$ as identified was inserted into the Sf9 cell vector pFASTBAC. Spinner cultures of Sf9 cells were infected with either the wild-type pFASTBAC ("Ctl) or pFASTBAC containing the 2.4-kb message ("γ"). At 48 hours post-infection, the cells were collected and cytosolic ("Cyto") and membrane ("Memb") fractions prepared as described above.

[000174] Sf9 cell cytosol and membrane fractions were loaded on a 10% polyacrylamide gel, resolved by SDS polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, by electroelution in 10 mM CAPS, pH 11, containing 10%methanol. Dry powdered milk (5% w/v) in 20 mM Tris-HCl, pH 7.4, containing 137 mM NaCl and 0.1% Tween 20 was used to block non-specific binding sites before incubation with

the primary antibody, immunoaffinity-purified antibody directed against iPLA$_2\gamma$, prepared as described below. Secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate) was incubated with the blot for 1 hour and immunoreactive bands were visualized by ECL.

[000175] Immunoaffinity-purified polyclonal antibody directed against iPLA$_2\gamma$ was prepared by immunizing rabbits with the iPLA$_2\gamma$ synthetic peptide CENLIPLDESRNEKLDQ (SEQ ID NO:26). This peptide (G3, 4 mg), dissolved in minimal volume of dimethylsulfoxide, was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH, 2 mg) in 200 td of 83 mM sodium phosphate, 0.9 M NaCl, and 0.1 mM EDTA at 22°C for two hours. After extensive dialysis against 83 mM sodium phosphate containing 0.9 mM NaCl, a 1:1 emulsion was made with the peptide-KLH conjugate and Freund's complete adjuvant. This emulsion was then injected subcutaneously into two anaesthetized New Zealand white rabbits. Two booster injections of a 1:1 emulsion of the peptide-KLH conjugate and Freund's incomplete adjuvant were given two and four weeks after the initial immunization, and then serum was collected. A peptide affinity column was generated by coupling the iPLA$_2\gamma$ synthetic peptide to a thiopropyl-Sepharose (Amersham-Pharmacia) resin in the presence of 100 mM sodium citrate, pH 4.5. The resin-peptide suspension was mixed by inversion overnight at 22°C. Coupling efficiency was monitored by the displacement of the 2-pyridyl sulfide groups as 2-thippyridone through spectrophotometric monitoring at 343 nm. Before chromatography, the resin-peptide suspension was equilibrated with 10 mMTris-HCl, pH 7.5 in an Econo-pac column (BioRad) by gravity flow. Serum containing the anti iPLA$_2\gamma$ antibodies was diluted 1:10 (v/v) with 10 mM Tris-HCI, pH 7.5 and then applied to the peptide affinity column by gravity flow. The diluted serum was then re-applied twice to the column to assure binding of the antibody to the column. After washing the column extensively with 10 mM tris-HCl, pH 7.5 containing 500 mM NaCl, bound anti-iPLA$_2\gamma$ antibody was eluted from the column with 100 mM glycine, pH 2.5 and the immediately neutralized with 100 mM Tris-HCl, pH 9.0. The antibody was then concentrated using Centricon-l0 (Americon) centrifugal filter devices and stored at 40°C until use.

[000176] Figure 15 shows the results of Western analysis of iPLA$_2\gamma$ expression in Sf9 cells, and confirm in an intact eukaryotic system the identification of translation initiation sites at methionine residues 101 and 221 as identified in the in vitro system as described in Example 4. The results as shown in Figure 15 are typical of three independent

experiments. Specifically, Western analysis of membrane fractions from Sf9 cells infected with vector harboring iPLA$_2\gamma$ ("γ Memb") demonstrated the presence of two major bands corresponding to molecular masses of 77 and 63 kDa. However, no such products were present in membrane fractions from Sf9 cultures infected with wild-type virus ("Ctl Memb"). In contrast, cytosolic fractions from control or iPLA2γ-transfected Sf9 cells did not contain any detectable immunoreactive protein. Collectively, these results support the existence of two translation initiation start sites at methionine residues 101 and 221.

EXAMPLE 6

[000177] To determine whether iPLA$_2\gamma$ translation products as described in Example 5 are biologically active, a phospholipase enzyme activity analysis was undertaken of Sf9 cells infected with recombinant baculovirus encoding iPLA$_2\gamma$. This example demonstrates that iPLA$_2\gamma$ translation products are biologically active and more specifically, are catalysts of cleavage of the sn-2-fatty acid of choline glycerophospholipids.

[000178] Briefly, Sf9 cells were infected with either wild-type baculovirus or baculovirus encoding human iPLA$_2\gamma$, as described in Example 5. Cytosolic and membrane fractions of the infected Sf9 cells were prepared as described above. Phospholipase A_2 activity was assessed as follows. Membrane fractions from control cells or cells expressing iPLA$_2\gamma$ were incubated with 40 μM L-α-1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine in 100mM Tris-Hac, pH 8.0, containing 1 mM EGTA at 37°C. Aliquots of the reaction were removed at the indicated times, and the amount of [1-^{14}C]oleic acid released was quantified as described above. Specifically, the release of sn-2-fatty acid from ~ glycero-3-phosphocholine was determined as a function of time.

[000179] Figure 16 is a graph showing an initial rate analysis of phospholipase activity in Sf9 cells infected with recombinant baculovirus encoding iPLA$_2\gamma$. The results shown in Figure 16 are representative of three independent experiments.

[000180] Membrane fractions from cells infected with vector harboring the iPLA$_2\gamma$ insert released fatty acid from the sn-2-position of phosphatidylcholine approximately 10-fold faster than from membrane fractions prepared control cells infected with wild-type vector. Liberation of radiolabeled fatty acid was nearly linear for 2 minutes at a velocity of approximately 0.3 nmol/mg-min. Phospholipase A_2 activity was entirely calcium independent in the range of 0-10 mM and demonstrated a pH optimum at pH 8.0 (not shown),

which is at or near physiologic pH. In contrast, phospholipase A_2 activities in cytosolic fractions of cells infected with wild-type vector and from cells infected with vector harboring the $iPLA_2\gamma$ insert were found to be similar (not shown).

[000181] To determine the substrate selectivity of the phospholipase A_2 activity demonstrated in Sf9 cells infected recombinant baculovirus encoding $iPLA_2\gamma$ multiple alternative phospholipase A2 substrates were tested. Membrane fractions from both groups of cells were incubated in 100 mM Tris-HCl, pH 8.0, containing 1 mM EGTA for 2 minutes at 37°C in the presence of either 40μML-α-l-dipalmitoyl-2-[1-^{14}C] phosphatidylcholine ("PPPC"), L-α-1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine ("POPC"), L-α-1-palmitoyl-2-[1-^{14}C] linoleoyl phosphatidylcholine ("PLPC"), or L-α-1-palmitoyl-2-[1-^{14}C] arachidonyl phosphatidylcholine ("PAPC"). Reactions were terminated by butanol extraction, and radiolabeled fatty acids were resolved by TLC and quantitated by scintillation spectrometry.

[000182] Figure 17 is a substrate selectivity profile of $iPLA_2\gamma$ phospholipase A_2 activity in Sf9 cells infected recombinant baculovirus encoding $iPLA_2\gamma$. Panel A of this figure shows the results of phospholipase A_2 activity analysis from Sf9 cells infected with either wild-type baculovirus (stippled bars) or recombinant baculovirus encoding $iPLA_2\gamma$ (solid bars). The results are representative of three independent experiments. Thus, incubation of membrane fractions containing $iPLA_2\gamma$ with phospholipids containing three distinct types of unsaturated fatty acids at the sn-2-position (oleic, linoleic, and arachidonic acids) gave similar specific activities that were each 6-10 times greater than activities manifest in membrane preparations derived from cells infected with wild-type vector.

[000183] Figure 17, Panel B shows the results of further phospholipase A_2 activity analyses undertaken to identify the initial site of hydrolysis of phospholipids by $iPLA_2\gamma$. Membranes derived from Sf9 infected with either wild-type baculovirus (stippled bars) or recombinant baculovirus encoding $iPLA_2\gamma$ (solid bars) were incubated with either 40μM L-α-1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine ("POPC"), or 40μM synthetic radiolabeled 1 -O-(Z)-hexadec- 1 '-enyl-2-[9,10-3H]octadec-9'-enoyl-sn-glycero-3-phosphocholine ("plasmenyl-PC"), under incubation conditions as described for Panel A. Plasmenyl choline has a relatively enzymatically resistant vinyl ether linkage at sn- 1.

[000184] Panel B of Figure 17 shows that the rate of hydrolysis using plasmenyl choline was similar to that using sn-2-[l-^{14}C]phosphatidylcholines, thus supporting the

conclusion that hydrolysis of substrate by iPLA2γ occurs predominantly at the sn-2-position. Therefore, $iPLA_2\gamma$ gene products are catalysts of cleavage of the fatty acid of choline glycerophospholipids.

EXAMPLE 7

[000185] To establish $iPLA_2\gamma$ as a member of the calcium-independent phospholipase $_{A2}$ family, the inhibition of the phospholipase A_2 activity of $iPLA_2\gamma$ by (E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL) was studied.

[000186] (E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL) has previously been shown to be a powerful and irreversible mechanism-based inhibitor of myocardial cytosolic and membrane-associated iPLA2, with nearly complete inhibition of myocardial cytosolic membrane-associated iPLA2 at concentrations of approximately 2-5μM. (Hazen et al., J. Biol. Chem. 266:7227-32, 1991; Zupan et al., J. Med. Chem. 36: 95-100, 1991; Gross et al., Biochemistry 32:327-36, 1993; Ramanadham et al., Biochemistry 32:337-46, 1993, which are herein incorporated by reference).

[000187] Briefly, Sf9 cells were infected with either wild-type baculovirus or baculovirus encoding human $iPLA_2\gamma$, as described in Example 5. Membrane fractions of the infected Sf9 cells were prepared as described above, and then incubated for three minutes at room temperature in 100 mM Tris-HAc, pH 8.0, containing 1mM EGTA, with varying concentrations of BEL, or ethanol vehicle, as indicated along the x-axis. L-α-1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine (40μM final concentration) in ethanol was then added to each reaction, followed by incubation at 37°C for two minutes. Released [1-^{14}C]oleic acid was quantitated as described above.

[000188] Figure 18 is a graph showing data of inhibition of the phospholipase A_2 activity of $iPLA_2\gamma$ by (E)-6-(bromomethylene)-3-(1 -napthalenyl)-2H-tetrahydropyran-2-one (BEL). The results shown in Figure 18 were representative of three independent tests.

[000189] As shown in Figure 18, preincubation of membranes harboring $iPLA_2\gamma$ with 5μM BEL for three minutes prior to the addition of substrate resulted in the inhibition of approximately 70% of $iPLA_2\gamma$ phospholipase A_2 activity. The IC50 for BEL inhibition of $iPLA_2\gamma$ was approximately 3μM. Thus, the results establish that BEL is an effective inhibitor of $iPLA_2\gamma$.

[000190] Previous work has characterized $iPLA_2$ activity in certain tissues, as well as a calcium-facilitated phospholipase $A_2\gamma$. Specifically, prior studies have identified $iPLA_2$ activity in cytosolic and membrane fractions of canine and human myocardium. (Wolf, R.A. and Gross, R.W., J. Biol. Chem. 260:7295-303, 1984; Hazen, S.L. and Gross, R.W., Circ. Res. 70:486-95, 1992, which are herein incorporated by reference). Additional studies have demonstrated an increase in membrane-associated, BEL-inhibitable $iPLA_2$ activity during myocardial ischemia or hypoxia. (Hazen et al., J. Biol. Chem. 266:5629-33, 1991; Ford et al., J. Clin. Inv. 88:331-35, 1991; McHowat J. and Creer, M.H., Lipids 33:1203-12, 1998; McHowat et al., Am. J. Physiol. 274:C1727-37, 1998, which are herein incorporated by reference). It has also been proposed that increased myocardial iPLA2 activity during ischemia contributes to ventricular arrhythmias and hemodynamic dysfunction through the production of lysophospholipids and arachidonic acid, each of which has potent electrophysiological effects. (Wolf, R.A and Gross, R.W., supra; Gross, Trends Cardiovasc. Med. 26:115-21, 1992; and Gross, Biochem. Soc. Trans. 26:345-49, 1984, which are herein incorporated by reference).

[000191] A calcium-facilitated phospholipase $A_2\gamma$ shares homology with the catalytic domain of calcium-facilitated phospholipase $A_2\alpha$ and has been cloned and expressed. Northern analysis has demonstrated the presence of calcium-facilitated phospholipase $A_2\gamma$ message in skeletal and heart muscle, and cloning and expression of calcium-facilitated phospholipase $A_2\gamma$ in Sf9 cells has confirmed the membrane localization and calcium-independence of calcium-facilitated phospholipase $A_2\gamma$. (Underwood et al., J. Bio. Chem. 273:21926-32,1998 which is herein incorporated by reference). However, calcium-facilitated phospholipase $A_2\gamma$ is not inhibited by BEL at $<50\mu M$ [BEL] (C.M. Jenkins, D.J. Mancuso, X. Han and R.W. Gross, "Identification of Calcium-independent Phospholipase A_2 ($iPLA_2$) β, and Not $iPLA_2\gamma$, as the Mediator of Arginine Vasopressin-induced Arachidonic Acid Release in A-10 Smooth Muscle Cells", The Journal of Biological Chemistry, Vol. 277, No. 36, Issue of September 6, pp. 32807-32814, 2002 which is herein incorporated by reference in its entirety) and is therefore clearly distinguishable from $iPLA_2\gamma$, which is subject to potent inhibition by BEL as shown in Figure 19. In contrast, the large majority of $iPLA_2\gamma$ activity from ischemic hearts or hypoxic myocytes in culture is membrane-associated and exquisitely sensitive to BEL inhibition. Accordingly, the inventors herein have succeeded in identifying $iPLA_2\gamma$ as a likely polypeptide for catalyzing the increase in ischemia-induced $iPLA_2\gamma$ activity. Further, the results suggest that the

peroxisomal compartment is a source of regulatory and potentially arrythmogenic eicosanoid metabolites and lysophospholipids.

EXAMPLE 8

[000192] To determine whether iPLA$_2\gamma$ expression is subject to translational control, multiple iPLA$_2\gamma$ truncation mutants were prepared and their expression in Sf9 cells studied.

[000193] To prepare truncated iPLA$_2\gamma$ constructs in pFASTBAC, PCR primers containing appropriate restriction sites for cloning were used, and performed as described above. For example, the original full-length 88 kDa construct was used as a template in PCR with a sense primer annealing at nt 650-676 paired with reverse primer M458 (5'-GCATAGCATGCTCACAATTTTGAAAAGAATGCAAGTCC-3') (SEQ ID NO:12) to create a truncated iPLA$_2\gamma$ (63kDapF) with a methionine start site corresponding to amino acid 221 in the full-length protein. Expression of this construct in the baculovirus system as described above resulted in a 63 kDa recombinant protein. A second construct was created with a start site corresponding to methionine 122 to produce a 74kDa recombinant protein. Additional constructs were also prepared as described above to produce N-terminal truncated recombinant proteins of approximately 77, 70 and 66kDa that were used in additional studies of translational inhibition as described below in Example 9. Further, the same methods were used to for segments of iPLA$_2\gamma$ 5' sequence ranging between 15-663 nt which were inserted 5' of the insert sequence in 63kDapF to identify potential sequences involved in translational regulation, as described below in Example 10.

[000194] The expression of the 63 kDa and the 74 kDa truncation mutants were studied in Sf9 cells using the expression system as described above. Cytosolic and membrane fractions were obtained as described above, and a Western analysis performed on the polypeptide products of the 63 kDa and 74 kDa truncation mutants of iPLA$_2\gamma$, as expressed in cytosolic and membrane fractions prepared from Sf9 cells.

[000195] Figure 19 shows the results of the Western analysis. Both cytosolic and membrane fractions ("63 kDa iPLA$_2\gamma$Cyto" and "63 kDa iPLA$_2\gamma$ Memb", respectively) from Sf9 cells infected with vector harboring truncation mutants coding the 63 kDa product of iPLA$_2\gamma$, demonstrated the presence of major bands corresponding to molecular mass of 63 kDa, though to a lesser extent in the cytosolic fraction. However, analysis of both cytosolic

and membrane fractions ("74 kDa iPLA$_2\gamma$Cyto" and "74 kDa iPLA$_2\gamma$Memb", respectively) from Sf9 cells infected with vector harboring truncation mutants coding the 74 kDa product of iPLA$_2\gamma$, demonstrated greatly decreased amounts of product in comparison to that expressed using the 63 kDa truncation mutant. Similarly, analysis of both cytosolic and membrane fractions ("iPLA$_2\gamma$Cyto" and "iPLA$_2\gamma$Memb", respectively) from Sf9 cells infected with vector harboring truncation mutants coding the iPLA$_2\gamma$ (i.e. wild-type 88 kDa protein), demonstrated little or no expressed product in comparison to that expressed using the 63 kDa truncation mutant. Collectively these results support the existence of a sequence or sequences involved in translation inhibition. Further, such sequences can be localized to the stretch of sequence representing the difference between the 63 kDa truncation mutant and the 74 kDa truncation mutant.

EXAMPLE 9

[000196] To more precisely localize the sequence involved in transcriptional and/or translational inhibition of iPLA$_2\gamma$, additional constructs (70kDa and 66kDa) were prepared utilizing the methods described above that were used for preparation of the 88kDa, 77kDa, 74kDa, and 63kDa in vector pFASTBAC for baculoviral expression. Unlike the 88kDa, 77kDa, 74kDa, and 63kDa constructs which correspond to authentic ATG start sites in iPLA$_2\gamma$, artificial ATG start sites were introduced to synthesize the 70kDa and 66kDa constructs. In brief, sense PCR primers were prepared which were downstream from the 74kDa start site and contained a restriction enzyme site (SalI) followed by an ATG start site. The primer for the 70kDa construct was M33636 (5'-AAAAGTCGACATGAAGCCATCAAATCTC-3') (SEQ ID NO: 55) corresponding to an ATG start site at nucleotide 452 of SEQ ID NO: 13. The primer for the 66kDa construct was M33637 (5'-AAGAGTCGACATGAGGTAAACGCAGTC-3') (SEQ ID NO: 56) corresponding to an ATG start site at nucleotide 549 of SEQ ID NO: 13. These primers was paired with M458 (SEQ ID NO: 12) to amplify products of 1.9kb and 1.8kb, respectively for the 70kDa and 66kDa constructs, from the full-length 88kDapF construct used as a template. PCR products were then cloned via SalI/SphI restriction sites into the polylinker region of pFASTBAC vector for use in the baculoviral expression system as described earlier. Figure 20 is a schematic illustration of each of the polypeptides products. The rectangular block at the top represents the full-length 88kDa iPLA2γwith the locations of alternative initiator methionine residues M1, M102, M122, and M221 indicated. Below this rectangle are represented each of the truncated constructs prepared, with the left side of the block

representing the degree of truncation in reference to the full-length 88kDa iPLA$_2\gamma$. The relative amount of activity of each of these constructs as assayed in the phospholipase A_2 assay described earlier is indicated on the left of each representation of the constructs. Essentially no or very little phospholipase A_2 activity was measured under the assay conditions used until iPLA$_2\gamma$ sequence was truncated to the 70kDa construct and high activity was measured for the 66kDa as well as for the 63kDa constructs. These results suggested that an inhibitory sequence lay in the region between the 5' start of the 74kDa and 70kDa constructs. This region is represented by the smaller brackets at the bottom of the figure. Further, no immunoreactive band was detectable from cells containing the 77kDa truncation mutant or the 88kDa full-length sequence. The larger brackets indicate the results of other studies in which the location of the inhibitory region was defined by using iPLA$_2\gamma$ sequence placed upstream of the luciferase gene in a vector construct to direct luciferase expression in transfected CV1 cells.

EXAMPLE 10

[000197] To further identify and locate the sequences involved in translational inhibition of iPLA$_2\gamma$, the effect on translation of several iPLA$_2\gamma$ 23-mer sequences 5' of the 63 kDa sequence was studied.

[000198] Briefly, sequences from iPLA$_2\gamma$ were inserted by ligation of 15-23-mer annealed phosphorylated oligonucleotide pairs 5' of full-length luciferase coding sequence which was cloned into pFASTBAC via NotI/XbaI restriction sites. Luciferase activity of recombinant protein produced in the Sf9 system was then measured using the Luciferase Assay System of Promega. (Wood, K.V., (1991) In: Bioluminescence and Chemiluminescence: Current Status, Stanley, P. and Kricka, L. eds., John Wiley and Sons, Chichester, NY, 543; and Luciferase Assay System Technical Bulletintus, #TB 101, Promega Corporation, 1996, which are herein incorporated by reference). Figure 21 is a schematic diagram showing ligation of 23-mer iPLA$_2\gamma$ sequence to luciferase coding sequence and cloned into pFASTBAC.

[000199] Figure 22 is a schematic representation of the location of the 23-mer sequences studied. Schematic representations of the 74 kDa protein product of iPLA$_2\gamma$ as well as the 70kDa sequence, are shown at the top for comparison. "1/2", "3/4". "5/6", and "7/8" each indicate successive 23-mer DNA sequences taken from coding region representing the difference between the sequences for a 70 kDa iPLA$_2\gamma$ protein and the 74 kDa protein.

The numbers 1-8 indicate the primers that were used to amplify each 23-mer piece. Constructs were prepared by fusing luciferase coding sequence to a one of the four alternative 23mer sequences from iPLA$_2\gamma$, and to the baculovirus promoter. Four alternative constructs, each containing one of the four different 23mer sequences from the 74 kDa iPLA$_2\gamma$ truncation mutant, were prepared and then expressed in Sf9 cells as described above. After 48 hours allowed for expression, cell extracts were prepared and then tested for level of expression using the luciferase assay system as described in detail above.

[000200] Figure 23 shows the phosphorylated oligo pairs for sequence between nucleotide 364-455 that is involved in translation inhibition of iPLA$_2\gamma$ in the luciferase expression system, thus identifying four pairs, 1/2", "3/4". "5/6", and "7/8" which each indicate successive 23-mer DNA sequences taken from coding region representing between the N terminus of the 70 kDa iPLA$_2\gamma$ protein and the 74 kDa protein.

[000201] Figure 24 is a bar graph showing luciferase assay results of the expression of three of the four different iPLA$_2\gamma$ constructs. The graph plots relative light units (RLU) emitted for each of three constructs containing a different 23mer sequence from iPLA$_2\gamma$ 74 kDa truncation mutant, as described above. The first construct contained the 23-mer sequence identified as "1/2" on the x-axis, the second construct, contained the 23-mer sequence identified as "5/6" on the x-axis, and the third construct contained the 23-mer identified as "7/8" on the x-axis. The 3/4 sequence did not amplify. The results, including little or no expression of the construct containing the 1/2 sequence, thus show that at least a portion of 1/2 is involved in translational inhibition of iPLA$_2\gamma$. In contrast, the construct containing 5/6 resulted in a relatively high level of expression of iPLA$_2\gamma$, showing 5/6 is unlikely to be involved in translational inhibition. The results for the third construct containing 7/8 showed an intermediate level of expression that was not significantly different from the 5/6 construct. Thus, 7/8 is not likely to be involved in translational inhibition of iPLA$_2\gamma$. Thus, these results show that iPLA$_2\gamma$ is subject to translational inhibition by the 1/2 23-mer piece taken from coding region representing the difference between the sequences for a 70 kDa iPLA$_2\gamma$ protein and the 74 kDa protein.

EXAMPLE 11

[000202] To better understand the role of iPLA2γ in an intact animal, mice over-expressing iPLA$_2\gamma$ were prepared by exploiting the cardiomyocyte specificity of the α-mhc promoter. Briefly, we have engineered SalI sites on the 5' and 3' ends of iPLA$_2\gamma$ by PCR.

The SalI digested fragment was cloned into SalI digested and alkaline phosphatase treated a α-mhc vector and sequenced in both directions. A Not I fragment containing the α-mhc promoter in tandem with the $iPLA_2\gamma$ sequence was submitted to the Washington University Neuroscience Transgenic facility for microinjection of DNA directly into the pronuclei of mouse (B6CBAF1/J) zygotes which will result in integration of the transgene into the mouse germ line.

[000203] After weaning, founder mice will be identified by PCR analysis of mouse tail DNA utilizing specific primers which amplify 600 nt product containing the $iPLA_2\gamma$ sequence. The founder mice will be bred with WT B6CBAF1/J mice. Offspring harboring the $iPLA_2\gamma$ sequence overexpressed in a cardiac myocyte specific fashion will be identified by tail DNA PCR analysis. Offspring harboring the gene will be bred and the degree of $iPLA_2\gamma$ overexpression will be determined by quantitative PCR, Western blotting, and fluorescent measurement of tissue sections.

[000204] Lipids were extracted from mouse myocardium (~50mg) by a modified Bligh and Dyer technique utilizing 50 mM LiCl in the aqueous layer in the presence of internal standards selected based on their lack of demonstrable endogenous molecular ions in that region(1-3). ESI/MS analysis was performed utilizing a Finnigan TSQ-7000 Spectrometer as previously described (1-3).

[000205] Surprisingly, phosphatidyicholine, ethanolamine glycerophospholipids, and plasmaogen levels in fed transgenic mice were each reduced by about 60% of similarly fed WT mice. (Fig. 26) Triacylglyceride levels of fed transgenic vs WT mice were moderately increased (1.7 fold). Upon fasting in WT mice, levels of phosphatidylcholine, ethanolamine glycerophospholipids, and plasmaogen were reduced by 17% with no significant change in triacylglyceride levels. In contrast, no significant alterations of phosphatidylcholine, ethanolamine glycerophospholipids, and plasmaogen levels were detected upon fasting of transgenic mice. However, triacylglyceride levels increased 10 fold resulting in a 2 fold increase in total lipid mass. This increase was 1.9x and 1.6x that of WT fasted and fed mice, respectively (Fig. 26).

[000206] Accumulation of triacylglyceride in myocardial tissue has been shown to be associated with increased risk of cardiac dysfunction (Unger and Orei FASEB J 15: 312-321, 2001) resulting in 'lipotoxic heart' disease. Diabetes is also associated with

increased triacylglyceride accumulation in the heart as well as alterations in lipid metabolism. The accumulation of triacylglycerides and associated membrane lipid alterations in iPLA$_2\gamma$ transgenic mice (particularly under fasting conditions) is anticipated to contribute to an increased susceptibility for development of cardiomyopathy in this animal model. Studies of iPLA$_2\gamma$ transgenic mice have relevance with regard to the functional role of iPLA$_2\gamma$ in living organisms. These studies imply that iPLA$_2\gamma$ plays an important role in maintenance of normal lipid homeostasis in the heart and that dysfunction of iPLA$_2\gamma$ may lead to a predisposition for disorders of lipid metabolism, development of cardiomyopathy, and susceptibility to diabetic cardiomyopathy. The iPLA$_2\gamma$ transgenic mouse should be a useful model for study of inherited and acquired lipid disorders and further analysis of the role of triacylglyceride accumulation in diabetic and other metabolic associated cardiomyopathy. The iPLA$_2\gamma$ transgenic mice will also be a useful model to explore therapies for disorders associated with accumulation of triacylglycerides in the heart. In particular, therapies directed toward modulation of myocardial iPLA$_2\gamma$ levels will be relevant.

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EXAMPLE 12

[000210] To examine the stability of iPLA$_2\gamma$ message expressed in the baculoviral system, spinners (100ml) were infected with equivalent volumes of each truncated viral iPLA$_2\gamma$ construct (m.o.i.= 1) and 48h later, actinomycin D was added to a concentration of 10μg/ml. Following actinomycin D addition, aliquots (2ml) were removed,

centrifuged to collect pellets, and placed on ice at 0, 15, 30, 60, 120, and 240mm. RNA was prepared from each pellet following quick freezing in liquid N2 following the RNeasy (Qiagen) protocol. Recovery of RNA was determined spectrophotometrically at 260nm. RNA samples (2 μg) were fractionated on a 10% polyacrylamide Lattitude RNA midi gel (BioWhittaker) and blotted onto a nylon membrane, cross-linked by exposure to a UV light source for 1.5 mm, and then baked at 85°C for 60 mm. After prehybridization in ExpressHyb hybridization buffer (Clontech) for 30min, the blot was hybridized 1h at 68°C with a full-length iPLA$_2\gamma$ random-primed [^{32}P]dCTP in a minimal volume of hybridization solution, and washed with 2x SSC containing 0.1% SDS twice for 30 min each, followed by two washes with 0.1x SSC containing 0.1% SDS for 40 min each at 50°C, as described in the manufacturer's instructions. Hybridized sequences were identified by autoradiography for 16h.

[000211] The 63kD construct expressed over 10 fold more iPLA$_2\gamma$ message than either the 88kD or 74kD constructs. Message levels appeared to be similarly stable over a 4h period (Fig. 27).

EXAMPLE 13

[000212] To study the regulated expression of in vitro translated truncated iPLA$_2\gamma$ constructs, PCR primers were designed for PCR amplification of sequences for the full length (88 kDa) and for each of the truncated constructs (77, 74 and 63 kDa) and cloning via EcoRV and NotI sites into the polylinker site of vector pEF vector (Invitrogen). The methodology used is the same as that described earlier in sections 129-132 and 198.

[000213] The truncated iPLA$_2\gamma$ constructs in vector pEF (Invitrogen) were used in a TnT Quick Coupled Transcription/Translation System (Promega) as described by the manufacturer Proteins were separated by SDS PAGE, the gels dried, and exposed to autoradiography.

[000214] Truncation resulted in enhanced expression of iPLA$_2\gamma$ in the mammalian in vitro expression system. Expression of truncated constructs 77kD, 74kD, 7OkD, and 63kD were over 10 fold greater than expression of the full-length 88kD construct (Fig. 29). These results, in concert with results from expression in the baculoviral expression system suggest the presence of an inhibitory sequence between the 88kD and 63kD ATG start sites.

Example 14

[000215] Identification of Transcriptional Regulatory Elements in the 5' Coding Region of $iPLA_2\gamma$. The observed differences in baculoviral expression patterns of the sequentially truncated $iPLA_2\gamma$ message and resultant polypeptide synthesis from the four AUG start sites suggested the presence of a transcriptional inhibitory sequence between nucleic acid residues corresponding to the 88kDa and 63kDa AUG start sites. To localize the regulatory domain upstream of the 63 kDa start site of $iPLA_2\gamma$ responsible for transcriptional repression, PCR primers in Table III were used to amplify segments containing 124 nt of sequence upstream of the $iPLA_2\gamma$ 63 kDa start site. All 3' PCR primers in Table III were designed to generate identical upstream Kozak (GCCACCATG) sequences upstream from the ATG start. In each case the sequence around the ATG start is"GCCAXCATG" (where 'X' is a 'C' nucleotide in all constructs except 83 which contains an 'A' nucleotide). In each case, PCR products were cloned into HindIII/NcoI restriction sites within the polylinker region of pGL3 promoter vector (pGL3P). Additionally, because of the presence of a naturally occurring NcoI site within the 83 construct, an AflIII restriction site was utilized at the 3' end of this construct (instead of NcoI) to generate a compatible cohesive end for cloning into the NcoI restriction site of pGL3P. Transient transfection of CV-1 cells with each of the inhibitory constructs was performed using Lipofectamine Plus (Invitrogen). For each transfection, 1-2 μg of luciferase reporter plasmid was cotransfected with 100 ng of pcDNA 3.1/myc-His/lacZ vector and β–galactosidase activity was measured utilizing the β–Galactosidase Enzyme Assay System (Promega) for normalization of results. Background measurements were uniformly low and cell survival was indistinguishable in all transfections performed. The cells were harvested 24 hr later for assay using the Luciferase Assay System (Promega). The luciferase assay was performed using the Luciferase Assay system from Promega following the manufacturer's protocol. Relative luminescence values were measured in a Beckman Scintillation counter with a wide open window. Through this approach, we sought to determine which elements in the 5' coding sequence acted as transcriptional repressors in a mammalian cell line. Constructs corresponding to each of the first four 123 nucleotide sequences encoding truncated sequences from the 5' of nucleotide 315 greatly inhibited luciferase expression (on average ~80%) while segments further 3' were not inhibitory in comparison with control vector ($p < 0.001$) (Fig 39).

[000216] Fig. 36 Identification of a Regulatory Domain Within the Coding Region of $iPLA_2\gamma$ Using a Luciferase Reporter Assay System. The bar graph indicates the relative fluorescence value (RFV) of $iPLA_2\gamma$–pGL3P constructs compared with unmodified pGL3P vector control used in the luciferase assay system. PCR primer pairs used to prepare each of the 88, 87, 83, 80, and 74 constructs are shown in Table I. Below is a representation of the locations and sizes of $iPLA_2\gamma$ sequence within each of the constructs in comparison with full-length $iPLA_2\gamma$ coding sequence (solid bar). The positions of the 88, 77, 74, and 63 kDa start sites is indicated above the bar. A representation of a construct is shown at the bottom with the locations of the SV40 promoter, the $iPLA_2\gamma$ insert, and luciferase gene indicated.

Example 15

[000217] Demonstration of a Nuclear Protein Binding Site within the Transcriptional Regulatory Region of $iPLA_2\gamma$. Gel shift analyses were performed with the Promega Gel Shift Assay System according to the manufacturer's specifications by using 2 μg of nuclear protein for each gel shift reaction. For analysis of the 5' transcription inhibitory region of $iPLA_2\gamma$, double-stranded CdXa oligonucleotide containing 5' $iPLA_2\gamma$, sequence and corresponding to a region containing CdXa transcriptional binding sites as predicted by Transfact computer program (5'-TATTAATCTGACTGTAGATATATATATTTACCTCCTTAGTAATGC-3' (SEQ ID NO: 104) was end-labeled with [-^{32}P]ATP by using T_4 polynucleotide kinase, as instructed by the manufacturer (Promega). Competition studies were performed by adding a 10-fold molar excess of unlabeled oligonucleotide or randomized control oligonucleotide (5'-TTGATAGTTATCTATTACAGTCTTCTTAGATTGAAACAA-3') (SEQ ID NO: 94) to the reaction mixture prior to the addition of radiolabeled probe. Reaction mixtures were analyzed on 4% nondenaturing polyacrylamide gels in 0.5Å~ TBE (89 mM Tris-HCl, pH 8.0; 89 mM boric acid; 2 mM EDTA) as the running buffer. Electrophoresis was performed at 100 V for 2 to 3 h, followed by drying of the gel at 80°C under vacuum and visualization of DNA-protein complexes by autoradiography for 12 to 18 h. For analysis of the pre-exon 2 promoter region, Gel shift analysis utilizing radiolabeled CdxA nucleotide (corresponding to residues 6-50 starting from the 88 kDa AUG codon) revealed a single shifted protein-radiolabeled DNA complex utilizing HeLa nuclear extract which was competed out with a 100-fold molar excess of unlabeled CdxA but not with control oligonucelotide (Fig 37).

[000218] Fig. 37 Gel Mobility Shift Analysis of the iPLA2γ Regulatory Domain. Gel shift utilizing CdxA 1 oligo. Lanes: 1) Negative control minus HeLa nuclear extract, 2) Positive control containing HeLa nuclear extract, 3) Competitive assay containing 10 fold excess CdXA1 oligonucleotide, 4) Noncompetitive assay containing 10-fold excess randomized control oligonucleotide.

Example 16

[000219] Tissue Specific Translational Regulation of $iPLA_2\gamma$ in Myocardium. Due to the obvious complexity of the regulation of $iPLA_2\gamma$ resulting from the combined presence of transcriptional regulation, complex mRNA processing and utilization of MyoD promotor elements, we recognized that current hypotheses relegating the role of $iPLA_2\gamma$ exclusively to peroxisomal lipid metabolism were likely limited in appropriate scope. In prior work, we identified robust amounts of calcium independent phospholipase A2 activity in the mitochondrial compartment of both canine and human hearts (). Moreover, we recognized early on that the $iPLA_2$ family of proteins had the potential for providing substrate for mitochondrial fatty acid oxidation by lipid hydrolysis, for regulating complex protein networks by generation of lipid signaling molecules(eicosanoids and lysolipids) and for modulating ion channel structure and function (). Accordingly, we considered the possibility that myocardium could process $iPLA_2\gamma$ in ways completely distinct from that manifest in liver, and that the large amounts of $iPLA_2$ activity we previously characterized in mammalian mitochondria (including human mitochondria) could result, at least in part, from tissue specific processing of the $iPLA_2\gamma$ gene. Subcellular fractionation of mouse and rat heart by differential centrifugation was performed essentially as described previously for rat liver (jan). In brief, rat heart was minced and then homogenized in three volumes (w/v) of homogenization buffer (0.25 M sucrose, 5 mM MOPS, pH 7.4, 1 mM EDTA and 0.1% (v/v) of ethanol, 0.2 mM dithiothreitol containing protease inhibitors (phenylmethylsulfonyl fluoride (0.2 mM), leupeptin (1 μg/ml), aprotinin (1 μg/ml) and phosphoramidon (15 μg/ml) using a Potter-Elvehjem homogenizer at 1000 rpm with 8-10 strokes. Homogenate was then centrifuged at 100 x *g* for 10 min to collect cellular debris. Nuclear fraction pelle was obtained following 1000 x *g* for 10 min of the supernatant. Heavy mitochondrial fraction pellet was obtained by centrifugato3000 x*g* for 20 min centrifugation of the light mitochondrial supernatant. Light mitochondrial fraction pellets were obtained by 25,300 x *g* or 70,000 x g for 20 min. Finally, 100,000 x *g* was utilized to produce a microsomal pellet

and cytosolic fraction. Differential centrifugation demonstrated that $iPLA_2\gamma$ cosedimented with mitochondria, in particular the light mitochondrial fraction (Fig38). Remarkably, the entire series of predicted high molecular weight $iPLA_2$ proteins were present in rat mitochondria corresponding to utilization of translation initiation sites encoding proteins with Mr=63,74,77, and 88kDa protein products. These results identified the tissue specific translational regulation of $iPLA_2\gamma$ in rat myocardium and identified the molecular identity of at least one $iPLA_2$ activity we previously characterized in mammalian mitochondria.

[000220] Fig. 38 Immunoblot Analysis of $iPLA_2\gamma$ in Subcellular Fractionations of Rat Heart. Equivalent subcellular fractions (100ug) of rat heart were subjected to SDS PAGE and analyzed by Western blotting. Lanes: 1, rat heart homogenate; 2, crude pellet; 3, heavy mitochondrial fraction; 4, light mitochondrial fraction; 5, A 70,000 x g light mitochondrial fraction; 6, Nuclear fraction. Molecular weight markers are indicated on the left and the relative migrations of the 88, 77, 74 and 63 kDa $iPLA_2\gamma$ (Mancuso et al, J. Biol. Chem. 275:9937 (2000); Tanaka, et al, BBRC 272:320 (2000))polypeptides are indicated by the arrows on the right.

Example 17

[000221] Inr sequences typically occur at the transcription start site in genes (). An (Inr) sequence with a consensus sequence of Py-Py-A-N-T/A-Py-Py was identified in the pre exon 2 promoter region of $iPLA_2\gamma$ (Fig. 39). Inr oligonucleotide (5'- GCG TCA CTT CCG CTG GGG GCG G -3') (SEQ ID NO: 77) was used in competitive competition while randomized control oligo 5'- GTGGCCGGGTGGTCCACCTCGG-3') (SEQ ID NO: 93) was used for noncompetitive competition in gel shift analysis. .Gel shift analysis provided evidence that a specific transcriptional regulatory protein binds to the Inr region in pre-exon 2 sequence (Fig 40). The second region of 11 nt in length is of unknown function and located near the previously identified start of exon 2 (,). It is interesting to note that this region of high conservation includes a "AG" dinucleotide and thus potentially could identify an intron/exon boundary.

[000222] Fig. 39. Alignment of Mouse, Rat, and Human Pre-exon 2 Sequence. Boxed regions indicate highly conserved regions between mouse, rat, and human. A putative Inr site is underlined. Previously reported 5' ends of the $iPLA_2\gamma$ (Mancuso et al, J. Biol. Chem. 275:9937 (2000): Tanaka et al, BBRC 272:320 (2000)) message are indicated (<).

[000223] Fig. 40. Competitive gel retardation analysis of iPLA$_2\gamma$ regions. Gel shift utilizing INR oligonucleotide. Lanes: 1) Negative control minus HeLa nuclear extract, 2) Positive control containing HeLa nuclear extract, 3) Competitive assay containing 10 fold excess INR oligonucleotide, 4) Noncompetitive assay containing 10-fold excess randomized control oligonucleotide. Results are representative of three separate Gel shift assays.

Example 18

[000224] Identification of MyoD Binding Sites (E-boxes) within the Pre-exon 1 Region of the iPLA$_2\gamma$ Gene. We noted the presence of sequence corresponding to three E-boxes containing the MyoD consensus binding sequence 5'-CAGGTG-3')SEQ ID NO: 61) within the 350 nt upstream of exon 1 suggesting that promoter activity may be myoD dependent. To substantiate the functional importance of this assignment, additional experiments were performed. MyoD vector was obtained from Michael Chin (Harvard Medical School) who used it to study MyoD-dependent activation of the myogenin promoter. This vector was used in the Promega luciferase assay system along with iPLA$_2\gamma$ promoter constructs containing 600nt sequence upstream of exons 1 or 2 inserted upstream via HindIII/NcoI sites into the promoterless vector pGL3E from Promega. Empty pGL3E vector and the SV40 containing promoter vector pGL3P were used as controls. Results were normalized to β-gal resulting from cotransfection with a LacZ vector. Cotransfection of CV1 cells with MyoD and pre-exon 1 containing vectors resulted in a 5 fold stimulation of promoter activity compared with MyoD + pGLE vector cotransfection (Fig 41). These results suggest the importance of iPLA$_2\gamma$ in muscle tissues.

[000225] Fig. 41. MyoD Stimulation of Promoter Activity in Pre-exon 1 Sequence of iPLA$_2\gamma$. CV1 cells were transfected with promoterless vector (pGLE) or with a construct in which pre exon 1 sequence is inserted upstream of the luciferase gene in pGLE (pre-exon 1). Luciferase expression measured as relative luminescence value was determined with (+) or without (-) cotransfection with myoD vector. $P < 0.01$. Sequence of the pre exon 1 promoter region is indicated below. Locations of E-boxes (myoD binding sites) are underlined.

Example 19

[000226] Identification of Mitochondrial Import Sequences in iPLA$_2\gamma$ and Demonstration of Their Functional Integrity. Recent increases in our understanding of

mitochondrial import machinery has led to the generation of computer algorithms which can accurately assess the potential for mitochondrial localization of a peptide with great accuracy and predictive probabilities. Using Psort II (http://psort.nibb.ac.jp/) and MitoProt II 1.0a4 (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) online, human iPLA$_2\gamma$ was predicted to have a high likelihood (>95%) of having an N-terminal mitochondrial localization signal. Since human iPLA$_2\gamma$ has multiple translation initiation sites resulting in proteins of 88 kDa, 77 kDa, 74 kDa, and 63 kDa, sequences for each of these forms were also utilized in the prediction programs. The identical cleavage site was predicted for the 88 kDa, 77 kDa, and 74 kDa isoforms, while the 63 kDa form was not predicted to have a mitochondrial cleavage site. The 74 kDa mitochondrial localization signal is MISRLAQFKPSSQILRK Δ VS... (SEQ ID NO: 58) where the triangle "Δ " indicates the cleavage site between residues lysine (K) and valine (V). To confirm the mitochondrial localization of the iPLA$_2\gamma$ polypeptide experimentally by yet another independent method, the widely accepted technique of dual image analysis overlay was employed. A commercially available marker for mitochondrial localization (pDsRed2-Mito, BD Biosciences) was employed which encodes mitochondrial targeting sequence of human cytochrome c oxidase fused to red fluorescent protein was utilized as a mitochondrial marker.. To prepare the iPLA$_2\gamma$-GFP fusion protein construct, site directed mutagenesis was used to make a construct expressing a fusion protein consisting of residues 1-17 of iPLA$_2\gamma$ 74 kDa polypeptide at the N-terminus of GFP. In brief, complementary phosphorylated oligonucleotides were synthesized encoding the predicted 17 amino acid mitochondrial signal of iPLA$_2\gamma$. The oligonucleotides were also designed so that, upon annealing, they generate appropriate terminal restriction sites for ligation to restriction sites at the 5' end of the GFP coding sequence in pEGFP vector, thus generating the appropriate fusion construct. Single or cotransfections of Hela cells with these vectors were performed. After 48 h, cells were fixed with 4% paraformaldehyde, coverslipped and fluorescence analyzed by confocal microscopy.

[000227] Fig. 42. Import of GFP into mitochondria mediated by an N-terminal iPLA$_2\gamma$ mitochondrial import signal. HeLa cells were transfected singly or cotransfected with 74GFP and DSRed2-Mito constructs and visualized by confocal microscopy. The co-localization of 74GFP and DsRED2-mito was revealed by the nearly identical staining patterns and by the yellow color resulting from combination of green and red fluorescence.

[000228] Dual transfection of HeLa cells with the leader sequence of the 74kDa polypeptide proximal to GFP (residues 1-32 of the 74 kDa product) and an authentic mitochondrial localization sequence (human cytochrome c oxidase in pDsRed2-Mito) demonstrated that the 74kDa leader sequence of $iPLA_2$ resulted in a green punctate pattern and a red punctate protein, respectively (Fig. 42). Critically, merging of the two images yielded an intense yellow punctate pattern indicative of mitochondrial localization mediated by the $iPLA_2\gamma$ mitochondrial leader sequence (Fig. 42). Thus, by three independent techniques, buoyant density gradient centrifugation, computer prediction of mitochondrial localization sequence(localization in silico), and colocalization of the $iPLA_2\gamma$ mitochondrial leader sequence-tagged green fluorescent protein with an authentic mitochondrial marker, the results demonstrate that $iPLA_2\gamma$ is localized, at least in part, to the mitochondrial compartment in myocardium in contrast to its predominant peroxisomal localization in liver. Moreover, these results demonstrate dual localization sequences in a single protein and thus identify a competition for newly synthesized $iPLA_2\gamma$ between mitochondrial localization(from its N terminal mitochondrial localization sequence) and peroxisomal localization (Fig. 42) (from its C terminal SKL peroxisomal localization sequence).

[000229] All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended to summarize the assertions made by their authors and no admission is made as to the accuracy or pertinency of the cited references or that any reference is material to patentability.

[000230] While the invention has been described in terms of various specific embodiments in the examples, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the claims which follow.